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RECOMBINANT BRICHOS DOMAINS DELIVERED OVER THE BLOOD-BRAIN BARRIER: A POSSIBLE WAY TO TREAT ALZHEIMER´S DISEASE

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Cover illustration: The illustration is based on "**The Ingenious Gentleman Don Quixote of La Mancha**", Spanish novel written by Miguel de Cervantes. It symbolizes the idea of a scientist (Don Quixote) pursuing a goal (fight and win the battle against Alzheimer´s disease). Don Quixote is armed and carries his shield (BRICHOS domains), aiming to defeat the toxic windmills with amyloid fibrils as blades that are present in the brain. The windmills are the enemies seen by Don Quixote as giants with long arms, and they represent destruction (the cause of the disease). Although we may be wrong in other people´s eyes, it is still our own pursuit that matters to us.
Original cover by Lorena Galán-Acosta.

Recombinant BRICHOS domains delivered over the blood-brain barrier: a possible way to treat Alzheimer´s disease
THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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In the loving memory of the strongest person I have ever met and my inspiration; my grandmother.

And dedicated to all women in science.

"We must have perseverance and above all confidence in ourselves. We must believe that we are gifted for something and that this thing must be attained."

Marie S. Curie

POPULAR SCIENCE SUMMARY OF THE THESIS

Brain diseases constitute about 35% of all human diseases. The most important and susceptible organ in the human body is the brain and thus, it needs a special care and protection. Many substances that cause no harm to other organs can be dangerous to the central nervous system. For example, some proteins that are present in the plasma, such as albumin, could cause inflammation. Hence, our brain is armed with strategic mechanisms of defense. We can say that the brain lives in a box made of bones with blood vessels that constitute a safety system that protects it and the whole central nervous system. This sophisticated protective structure is known as the blood-brain barrier (BBB). The BBB is a natural defense formed by specialized cells called endothelial cells that form a wall restricting the movement of substances. It is essential for survival and it also becomes our worst enemy when it comes to treating certain diseases. For decades of clinical neurobiology, this barrier has represented a complication for the drugs to access the brain and therefore, an obstacle for those drugs that could help us to address the treatment of neurological diseases, among them Alzheimer´s disease (AD).

AD is the most frequent cause of dementia and even the most positive prognostics say that the number of people affected will increase in the incoming years. This disease represents one of the main challenges of current medicine and until now, there is no effective treatment that polish off the roots that cause it. The source of the problem lays in the accumulation of two substances that end up damaging the brain. One of them is called amyloid-beta (Aβ), which forms dense clusters called senile plaques, that cause toxicity to the brain. Therefore, eliminating these plaques and/or their toxic consequences is one of current strategies to fight the disease.

Aware of this problem, the core objective of this thesis has been to explore the possibility and efficacy of using BRICHOS proteins as a strategy for AD. We have previously shown that BRICHOS can prevent formation of toxic Aβ assemblies. In this context, the first aim was to test if BRICHOS produced in bacteria reach the brain when it is administered via injections in the tail vein of mice. Secondly, we employed a technology called focused ultrasound with microbubbles (MBs) made of lipids and gas, that opens the BBB only where and when it is applied, allowing BRICHOS to pass into the brain. Next, we explored the effects the MBs alone have on the ability for BRICHOS to cross the barrier. Finally, this work shows the efficacy of BRICHOS treatment in a mouse model of AD. Along these studies we have made two main relevant discoveries: the anti-amyloid BRICHOS can reach the brain in mice and the BBB passage is improved by MBs alone, and it also has positive effects on AD pathology in mice. This is written with the hope that it may open multiple small windows in the journey of finding the way out of this devastating disease.

ABSTRACT

One of the current major challenges to treat neurological diseases is the ability of drug candidates to cross the blood-brain barrier (BBB). Alzheimer´s disease (AD) is the most common form of dementia worldwide, affecting more people every year due to a world where the average lifespan is increasing. During the last decade, there have been many drug candidates in AD clinical trials. However, all of them have failed and poor permeability over the BBB is probably a contributing factor. The aggregation of the amyloid-β (Aβ) peptide is considered one of the trigger mechanisms of the disease, and Aβ constitutes the core of the amyloid plaques in AD brains. Thus, targeting Aβ seems like a relevant therapeutic approach. Previous studies have demonstrated the ability of recombinant human (rh) BRICHOS domain to efficiently delay the fibril formation of Aβ peptide and to reduce its neurotoxic effects, both *in vitro* and *in vivo*.

The general aim of the present thesis was to test the potential of rh BRICHOS domain as a therapeutic candidate for AD. In **paper I** we tested the ability of rh Bri2 and proSP-C BRICHOS domains to cross the BBB and their permeability over the blood-cerebrospinal fluid (CSF) barrier after systemic administration in the tail vein of wild type (wt) mice. We thus uncovered qualitatively different BBB permeabilities of rh Bri2 and proSP-C BRICHOS domains. Only rh Bri2 BRICHOS reaches the brain parenchyma while both proteins are detected in the CSF. In order to enhance the passage into the brain of intravenously administered rh BRICHOS domains, in **paper II** we used focused ultrasound (FUS) in combination with microbubbles (MBs) to transiently open the BBB in a specific region in the mouse brain. This led to efficient delivery of rh proSP-C BRICHOS over the BBB in the targeted hemisphere and, unexpectedly, to detection of rh Bri2 BRICHOS domains in both targeted and non-targeted brain hemispheres. Both rh proSP-C and Bri2 BRICHOS domains were internalized by a subset of neurons in the hippocampus and cortex. Based on the observations from paper II, in **paper III** we explored the effects of MBs without FUS on the BBB permeability of rh Bri2 BRICHOS domain fused to a tag or to a globular protein. MBs facilitate the passage of rh Bri2 BRICHOS domain and the fusion partners to the brain parenchyma. Finally, we evaluated treatment effects of a designed monomeric mutant, rh Bri2 BRICHOS R221E, in Aβ precursor protein (APP) knock-in mouse models of AD (**paper IV)**. Our results showed an improvement in working and recognition memory, a decrease in the plaque load, reduced astrogliosis and microgliosis, as well as a reduction in the amounts of astrocytes surrounding the Aβ plaques after repeated injections of rh Bri2 BRICHOS R221E.

These findings highlight that rh Bri2 BRICHOS domain, can enter the brain parenchyma and have positive effects in an AD model with advanced pathology. These results support continued investigations of Bri2 BRICHOS domain as a treatment strategy for AD.

LIST OF SCIENTIFIC PAPERS

The content of the doctoral thesis is based on the following papers referred to as **I-IV** in the text.

I. **Blood-brain and blood-cerebrospinal fluid passage of BRICHOS domains from two molecular chaperones in mice**.
Tambaro S., **Galan-Acosta L**., Leppert A., Chen G., Biverstål H., Presto J., Nilsson P. and Johansson J.
Journal of Biological Chemistry (2019); 294(8): 2606-2615.

II. **Recombinant BRICHOS chaperone domains delivered to mouse brain parenchyma by focused ultrasound and microbubbles are internalized by hippocampal and cortical neurons**.
Galan-Acosta L., Sierra C., Leppert A., Pouliopoulos A.N., Kwon N., Noel R.L., Tambaro S., Presto J., Nilsson P., Konofagou E.E. and Johansson J.
Molecular and Cellular Neuroscience (2020); 105, 103498.

III. **Bri2 BRICHOS domain passage over the blood-brain barrier is increased by intravenous lipid microbubbles**.
Galan-Acosta L., Manchanda S., Pouliopoulos A., Biverstål H., Kwon N., Noel R.L., Tambaro S., Presto J., Winblad B., Konofagou E.E., Nilsson P. and Johansson J.
Manuscript.

IV. **Treatment with a molecular chaperone designed against in vitro amyloid-beta toxicity attenuates pathologies in Alzheimer mouse model**.
Manchanda S., **Galan-Acosta L**., Abelein A., Tambaro S., Chen G., Nilsson P. and Johansson J.
Manuscript.

ADDITIONAL PAPERS NOT INCLUDED IN THE THESIS

V. **The synthesis and characterization of Bri2 BRICHOS coated magnetic particles and their application to protein fishing: Identification of novel binding proteins.**
Tigro H., Kronqvist N., Abelein A., **Galan-Acosta L.**, Chen G., Landreh M. Lyashkov A., Aon M.A., Ferrucci L., Shimmo R., Johansson J. and Moaddel R.
Journal of Pharmaceutical and Biomedical Analysis (2021); 198:113996.

VI. **Amyloid-β peptide targeting peptidomimetics for prevention of neurotoxicity**.
Honcharenko D., Juneja A., Roshan F., Maity J., **Galan-Acosta L.**, Biverstål H., Hjorth E., Johansson J., Fishan A., Nilsson L. and Strömberg R.
ACS Chemical Neuroscience (2019);10(3):1462-1477.

VII. **BRICHOS -an anti-amyloid chaperone: evaluation of blood-brain barrier permeability of Bri2 BRICHOS**.
Tambaro S., **Galan-Acosta L.**, Leppert A., Presto J. and Johansson J.
Amyloid (2017); 24(supl):7-8.

VIII. **Activation of chaperone-mediated autophagy as a potential anticancer therapy**.
Galan-Acosta L., Xia H., Yuan J. and Vakifahmetoglu-Norberg H.
Autophagy (2015); 11(12):2370-1.

IX. **Degradation of HK2 by chaperone-mediated autophagy promotes metabolic catastrophe and cell death.**
Xia H.G., Najafov A., Geng J., **Galan-Acosta L**., Han X., Guo Y., Shan B., Zhang Y., Norberg E., Zhang T., Pan L., Liu J., Coloff J.L., Ofengeim D., Zhu H., Wu K., Cai Y., Yates J.R., Zhu Z. Yuan J. and Vakifahmetoglu-Norberg H.
Journal of Cell Biology (2015); 210(5):705-16.

X. **Glucose metabolism provide distinct prosurvival benefits to non-small cell lung carcinomas.**
Wu R., **Galan-Acosta L**. and Norberg E.
Biochemical and Biophysical Research Communications (2015);460(3):572-7.

CONTENTS

LIST OF ABBREVIATIONS

Aβ	Amyloid-β
AD	Alzheimer´s disease
ADAM	A disintegrin and metalloprotease
AICD	APP intracellylar domain
APOE	Apolipoprotein E
APOJ	Apolipoprotein J
APP	Amyloid precursor protein
Asp	Aspartic acid
BACEC	Beta-Site APP-cleaving Enzime1; β-secretase
BBB	Blood-brain barrier
BCSFB	Blood-cerebrospinal fluid barrier
CLU	Clusterin
CNS	Central nervous system
Cys	Cysteine
ELISA	Enzyme-linked immunosorbent assay
EPM	Elevated plus maze
ER	Endoplasmic reticulum
FBD	Familial British Dementia
FC	Fear conditioning
FDD	Familial Danish Dementia
FUS	Focused Ultrasound
HSP	Heat-shock protein
IHC	Immunohistochemistry
IMAC	Immobilized metal affinity chromatography
ITM2B	Intergral membrane protein 2B
LPS	Lipopolysaccharides
mAb	Monoclonal antibody
MB(s)	Microbubble(s)
MRI	Magnetic resonance imaging
MWM	Morris water maze

NMDA	N-methyl-D-aspartate
NFT	Neurofibrillary tangles
NOR	Novel object recognition
OF	Open field
PD	Parkinson´s disease
ProSP-C	Prosurfactant protein-C
PSEN	Presenilin
Rh	Recombinant human
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEC	Size exclusion chromatography
SP-C	Surfactant protein C
TM	Transmembrane
UPR	Unfolded protein response
wt	Wild type

1 INTRODUCTION

" Take care of all your memories. For you cannot relive them." -Bob Dylan

Alzheimer´s disease (AD) is a rising problem affecting around 70% of people suffering from dementia. This terrible type of dementia was defined for the first time during a lecture given by the German psychiatrist Dr. Aloysius Alzheimer in 1906 (1). During his lecture Alzheimer described his patient, called Auguste Deter, who was suffering from a cluster of symptoms: hallucinations, delusions, progressive cognitive impairment, and psychosocial incompetence.

"…What year is it? *Eighteen hundred.* Are you ill? *Second month.* What are the names of the patients? She answered quickly and correctly. What month is it now? *The 11th.* What is the name of the 11th month? *The last one, if not the last one.* Which one? *I don´t know*… When did you marry? *I don´t know at present. The woman lives on the same floor.* Which woman? *The woman where we are living.* I show her a key, a pencil and a book and she names them correctly. What did I show you? *I don´t know, I don´t know.* It´s difficult, isn´t it? *So anxious, so anxious*…"

Dr. Aloysius Alzheimer, Extracts from November 29, 1901.

After four and a half years of illness, Auguste died and Dr. Aloysius Alzheimer could observe the atrophy occurring in her brain.

"*Further examination shows many fibrils located next to each other which have been changed in the same way. Next, combined in thick bundles, they appear one by one at the surface of the cell. Finally, the nucleus and the cell itself disintegrate and only a tangle of fibrils indicates the place where a neuron was previously located… Approximately 1/4 to 1/3 of all neurons of the cortex show these changes. Many neurons, especially the ones in the upper layer, have completely disappeared"* (1).

For many years the symptoms of AD were considered a natural effect of aging. More than 100 years after the first report, and thanks to the development of molecular biology techniques such as DNA sequencing, it was confirmed that the patient described by Dr. Alzheimer carried a mutation in the presenilin (PSEN1) gene, associated with early-onset familial AD (2). Currently, we know that what Dr. Alzheimer observed in brain tissue comprises aggregated amyloid protein, but a full understanding of the molecular mechanisms of AD is still lacking (3). The main pathological features of AD are the formation of amyloid plaques of amyloid-β (Aβ) peptide and formation of neurofibrillary tangles from the tau protein. Despite all the intensive studies in the field, the exact events that cause the generation of those aggregates, and importantly, how they give rise to neurotoxicity and degeneration, remain unknown. However, much data support that disturbances of Aβ metabolism are involved in the pathogenesis (4). To the date the amyloid cascade hypothesis that was postulated in 1990´s has dominated the

research (5) and based on that, the inhibition of Aβ aggregation and toxicity are tempting strategies to counteract AD. However, treatment of AD is a complex problem that has not been resolved and there are many challenges that we face regarding the treatments targeting Aβ.

In an AD brain, there are alterations in the amounts of many different proteins and the BRICHOS domain from Bri2 proteins is one of them (6). This domain was characterized for the first time by Luis Sanchez-Pulido et al. in 2002 (7) and later shown to be able to bind the β-sheet aggregation-prone region of their proproteins, preventing the formation of amyloid-like fibrils (8-11). Since then, a number of studies have been done on the BRICHOS domain that show its role in efficiently inhibiting Aβ aggregation and toxicity both *in vitro* (8, 10, 12-16) and in animal models (17, 18). Hence, we consider BRICHOS domain to be a novel anti-amyloid candidate for AD treatment.

In this thesis, I have aimed to add a small piece to the big and complicated puzzle that constitutes AD research. My overall goal has been to understand if recombinant human (rh) BRICHOS domains could be used to treat AD. First, I have focused my studies on understanding if rh BRICHOS domains could pass to the brain parenchyma when they are peripherally administered. Further, I have studied two ways to facilitate the delivery of rh BRICHOS domains to the brain. And lastly, my approach has been to study whether a designed monomeric mutant of rh Bri2 BRICHOS domain (19) can be used to treat two mouse models that exhibit AD-like pathology.

2 BACKGROUND

" Every brilliant experiment, like every great work of art, starts with an act of imagination."
-Jonah Lehrer

2.1 FROM PROTEIN TO DISEASE

We often hear the word protein and associate it with the nutrients present in the food we eat, but they are also microscopic machines and indispensable components of cells in every living organism. The completion of the Human Genome Project has shown that the world of proteins is highly diverse in terms of function and structures. It was the Swedish chemist Jöns Jacob Berzelius who first suggested the term protein derived from *proteios*, meaning first position, to name these substances. Nowadays, we know that proteins constitute about 20% of the total body mass (20). According to the human genome data, it is estimated that around 19,200 proteins are expressed (21).

Proteins are synthesized by the ribosome from a total of 20 amino acids that can be arranged into different combinations, from small peptides to long proteins composed of thousands of amino acid residues. Proteins play a key role in regulating and maintaining virtually all biological reactions in the cell, allowing replication of the genetic information, transportation of molecules, eg oxygen, providing support and immune protection and many more events to happen. Their specific work is defined by their shape and structural changes, and to provide their functions, proteins need to be folded in a correct conformation. Given this, it is not strange that a failure in folding of proteins in the cell result in problems or even diseases. One of those is Alzheimer´s disease.

2.1.1 Basics on protein structure

Proteins are molecular machines and to fulfill this role, they need to first fold into a correct three-dimensional structure. The first level of arrangement is called primary structure. It consists of the sequence of amino acid residues ordered and held together via peptide bonds that form a linear chain. The peptide bond involves an α-carboxyl group of one amino acid and an α-amino group of the next. Each amino acid residue has a side chain linked to the α-carbon, which impacts on biochemical and biophysical properties and interactions with other molecules. But most proteins are not only a linear chain of amino acid residues; instead, they are folded into complex structures.

The two types of secondary structure, stabilized by hydrogen bonds between the amide and carbonyl moieties of the peptide bonds, are the α-helix and the β-sheet, proposed for the first time by Linus Pauling and co-workers (Figure 1) (22). The α-helix results in a twisted chain of residue side-chains pointing outwards in a helical array. It is stabilized by hydrogens bonds

between the carbonyl oxygen of residue *i* and the amide hydrogen four residues ahead (*i+4*) in the sequence, giving 3.6 amino acid residues per full turn. The β-sheet is formed by two or more polypeptide chain segments, β-strands, that are linked by hydrogen bonds between backbone amides and carbonyls (23). The strands of the β-sheets can run in opposite (antiparallel) or same (parallel) direction, but there can also be a mix of both types of strands, as for example found in the BRICHOS domain.

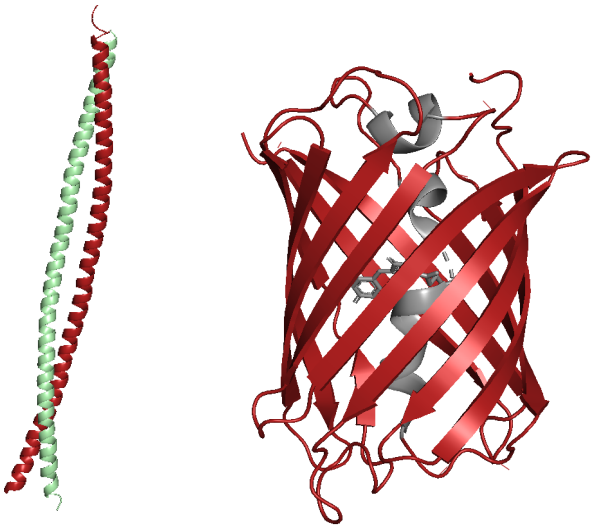

Figure 1. **Examples of an α-helix and a β-sheet conformation**. On the left, the crystal structure of α-keratin (PDB: 6JFV), consisting of two parallel α-helices (red and green colors) twisted around each other to form a coiled coil. On the right, the crystal structure of the fluorescent protein mCherry (PDB: 2H5Q) consisting of 3 α-helices (grey color) and 13 antiparallel β-strands (red color) forming a barrel structure.

The tertiary structure defines the three-dimensional organization of the folded polypeptide, maintained by hydrophobic interactions, van der Waals forces and other electrostatic interactions and covalent disulfide bonds between side chains as well as other parts of the polypeptide chain. The most common tertiary structure is a globular arrangement, where the hydrophobic parts are facing the core and the hydrophilic ones are oriented towards the surface, interacting with water. Moreover, a variable number of different (heteromers) or the same (homomers) protein chains can interact with each other, thus forming the quaternary structure. The sequence of amino acid residues determines the three-dimensional conformation and, therefore, the function.

2.1.2 Protein folding and proteostasis

Two scientists led the foundation of protein folding field. The first was Anfinsen, who demonstrated that the native state of a protein occurs at a local minimum of energy determined by the amino acid sequence (24). And in 1968, Levinthal pointed out the kinetic paradox indicating that folding cannot occur by stochastic exploration of all the possible conformations, as this would require enormous time for a protein to fold, but also the folding pathway of a protein is coded in the amino acid sequence of the polypeptide chain. Levinthal determined that there are intermediates formed during the folding process, and the number of possible

conformations populated before the protein reaches its native state is thus limited (25). *In vivo*, proteins start to acquire their three-dimensional native structure while they are leaving the ribosome, but they also fold post-translationally (26). The folding events allow a protein to obtain its functional shape, but it is a complex process that sometimes fails. The crowded environment inside the cell (27), occupied by proteins at a concentration estimated to be >300 g/L (28), makes this process even more complicated.

In eukaryotic cells, protein folding occurs not only co-translationally but also after passage into different organelles and cells are prepared with a quality control system, in the distinct cellular compartment where folding occurs, to ensure protein homeostasis (Figure 2). This quality control system influences protein aggregation, degradation and trafficking. Some of the mechanisms that cells use to prevent aggregates include the presence of molecular chaperones that accompany other proteins through their folding to avoid this process to fail (29). Chaperones are themselves proteins, and it is estimated that there are nearly 200 chaperones and co-chaperones cooperating to maintain the integrity of the proteome. Molecular chaperones recognize unfolded parts of proteins, typically exposed hydrophobic segments, and assist in protein refolding by preventing protein-protein interactions that may result in aggregation (30, 31). Additional factors, eg changes in the translation speed of the nascent polypeptide by, for example, the incorporation of rare codons have evolved to prevent protein misfolding and aggregation (32). The molecular chaperones have an essential role in the regulation of the proteostasis in cells, thus preventing disease or cell death. The molecular chaperones include the heat shock protein (HSP) families: HSP40, HSP60, HSP70, HSP90 and HSP110. Notably, molecular chaperones have been detected around plaques in AD and together with α-synuclein deposits in Parkinson, perhaps as a result of failed attempts to prevent aggregation (33) and can influence neurodegenerative diseases (34, 35). The extracellular chaperone apolipoprotein J (APOJ) also called Clusterin (CLU), has been identified as a modulator of Aβ toxicity (36) and has been observed to co-localize with amyloid plaques in AD (37). In addition, the molecular chaperone domain BRICHOS, also found to co-localize with plaques in AD (6), has been shown to have an anti-amyloidogenic activity (7, 9, 12, 13, 38, 39). The BRICHOS domain and its activity against Aβ aggregation will be described in more detail in section 2.4. Besides the critical role of chaperones in proteostasis, there are additional pathways adapted to avoid protein aggregation. These pathways include the cytosolic stress response and the unfolded protein response (UPR) in the endoplasmic reticulum (ER). If these fail, cells deal with the degradation of misfolded or aggregated proteins through the ubiquitin-proteasome system (UPS), ER-associated degradation, or the autophagy-lysosome system (40).

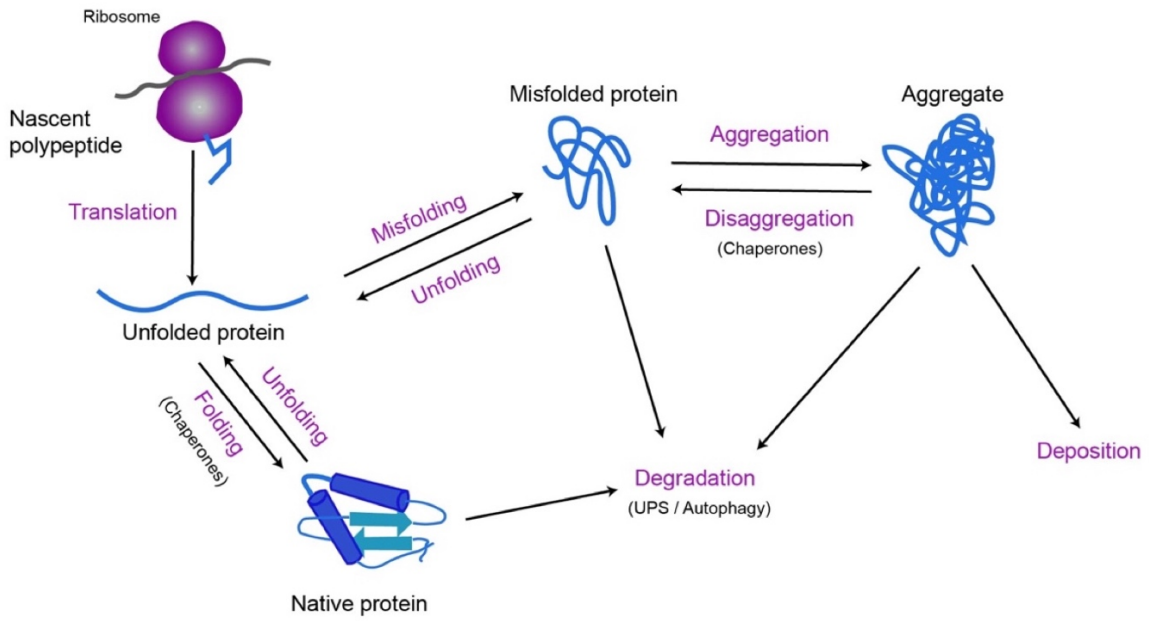

Figure 2. The proteostasis network. Schematic figure of the cellular mechanisms involved in protein quality control to ensure proteome stability. Protein production in cells involve a series of steps: transcription, translation, protein folding -facilitated by chaperones-, transport and degradation that needs to be maintained in balance. If any event causes the quality control to fail, it can lead to accumulation of misfolded proteins that are either sent for degradation or accumulated in deposits consisting of protein aggregates.

Protein aggregation and accumulation of misfolded proteins is constantly monitored, but protein homeostasis can be compromised with age, and protein aggregation is a well-recognized hallmark of several neurodegenerative diseases. During ageing the defense mechanisms probably start to weaken, and protein misfolding can thus overwhelm clearance, resulting in formation of protein aggregates. In support of this idea, reduction of molecular chaperones and alterations of proteasome activity have been found in aged rodents and human cells (41). The toxic aggregates that accumulate in cells cause stress that may further impair folding and clearance mechanisms (42). Neurons are especially vulnerable to aggregated and misfolded proteins since they cannot eliminate or dilute toxic molecules by division (43). The exact mechanisms that cause disease are likely complex, including loss of function as well as toxic gain of function caused by accumulation of aggregates. It is believed that soluble oligomeric aggregates formed during the fibril formation pathway are the primary toxic species. One supporting hypothesis suggests that the oligomers expose hydrophobic surfaces and mediate atypical interactions in the cell (44). In addition, it has been shown that protein aggregates could cause toxicity by forming pore-like structures that disintegrate the cell membrane (45). Irrespective of underlying mechanisms, the presence of fibrillar deposits of a specific protein or peptide is the defining feature of the amyloid diseases.

2.1.3 Amyloid and amyloid diseases

The term amyloid was coined in 1842 by Matthias Schleiden who referred to starch (from *amylum* in Latin). Later, in 1854, Rudolph Virchow introduced the term amyloid when he studied *cerebral corpora amylacea*, which had an abnormal structure that he considered to be

starch. Later, it was demonstrated that the substances observed were a “mass” of protein that had undergone structural changes that result in the formation of fibrils (46).

Almost any protein, independent of properties like e.g., native structures, can enter an amyloid state, whereby they form elongated and insoluble fibers consisting of β-sheets (47, 48). The propensities to form amyloid fibrils are, however, dependent on the exact amino acid sequence, and several amyloid diseases only occur, or are overrepresented, in the presence of certain mutated versions of the amyloidogenic protein. Initially it was thought that amyloid was composed of amorphous structures; but already studies using polarized light microscopy demonstrated a common structural motif to all amyloids (46). Amyloid fibrils have a characteristic cross-β structure composed of β-sheets running parallel to the long axis of the fibril, and consequently the β-strands are oriented perpendicularly to the fibril axis (49, 50). The histopathological definition of amyloid is based on staining with the dye Congo Red and birefringence when Congo positive deposits are viewed under crossed polarizers. Initially amyloid was defined as exclusively extracellular but it is now recognized that also intracellular amyloid deposits exist (51-53). The typical mature fibrils have a diameter of 2-10 nm and can be several micrometers long (54). Because of their order and arrays of inter-strand hydrogen bonds, amyloid fibrils are highly stable structures (55).

According to the previously mentioned definition, more than 35 amyloid diseases have been identified in humans, which are associated with specific proteins that can form extra- or intracellular deposits, and some proteins can give rise to more than one amyloid disease (47, 52, 56). Some examples of amyloidogenic peptides and proteins are Aβ, involved in AD (57), surfactant protein-C (SP-C) present in amyloid found in interstitial lung disease (ILD) associated with mutations in proSP-C (58), ABri and ADan peptides derived from mutant Bri2 protein, that are involved in familial British and Danish dementia (FBD and FDD) (52), polyQ expanded huntingtin and α-synuclein associated with Huntington disease and Parkinson´s disease (PD), respectively (56). In the case of Aβ, point mutations in its sequence (59), in the Aβ precursor protein or processing enzymes, and possibly changes in post-translational modifications (i.e. phosphorylation) (60) can lead to manifestation of amyloid. The amyloid deposits can be found either in one site (localized amyloid) or in multiple places (systemic amyloid) (52). However, not all types of amyloid deposits are pathological: so called functional amyloids can be found in bacteria, fungi, yeast and even humans, and are involved in various processes like the formation of biofilms, melanin synthesis, spider silk formation (61) or the storage of peptide hormones (62, 63).

2.2 ALZHEIMER´S DISEASE

One of the focus of this thesis is AD, in particular how the disease can be targeted by interfering with toxic consequences of protein aggregation. This chapter is focused on giving a general overview of AD, with emphasis on the role of Aβ.

2.2.1 Pathogenesis of AD

AD is a fatal neurodegenerative disease that causes progressive degeneration of neuronal cells, resulting in cognitive decline (64). AD is the most common cause of dementia, constituting about 50-70% of all cases world-wide (65). The prevalence of AD increases with age, which is the most important risk factor: AD occurs in 3% of individuals aged 65 years or younger, while about 32% of individuals aged 85 years or older have AD (66). As life expectancy is increasing world-wide, the number of people suffering from AD related dementia is expected to rise. Today an estimated 40 million people have dementia, and this is predicted to double over the next 20 years (67). Increased Aβ levels can begin to develop at least 2 decades before cognitive AD symptoms are noticed (5), suggesting that the number of AD patients would be significantly higher if diagnostic methods would allow earlier diagnosis than today. Patients with AD experience multiple symptoms that change over time, probably reflecting the progressive nature and spread of neuronal damage (68). An early and common symptom is difficulties in remembering recent events, and as AD progresses, the patients can develop disorientation, confusion, mood swings, behavioral changes, difficulties in speaking (aphasia), and problems with performing practical actions (apraxia). The progressive cognitive deterioration affects the daily life of patients as well as their families (65, 69). AD not only affects individuals and relatives, but also has a big economic impact on society (69). The financial cost of AD is high due to the lack of autonomy that the patients have, and the extensive social and medical help that they need to face the disease.

In the 80s, two independent groups found that the amyloid component of cerebrovascular amyloid and neuritic plaques is a small peptide fragment, today now known as Aβ (70, 71). A few years later, it was clarified that Aβ is the cleavage product of a membrane-bound protein with largely unknown function. The gene encoding the amyloid precursor protein (APP) is located on chromosome 21. This explains why individuals with trisomy 21 (Down´s syndrome) show increased plaque load and elevated risk of developing AD (72). Because of its association with AD, it was thought that Aβ is an abnormal cleavage product, but it was soon discovered that it is continuously produced as a soluble peptide also during normal cell metabolism (73, 74). Nearly a dozen of different types of amyloid deposits has been described in AD brains, but the two most widely observed can be classified as diffuse (immature) or neuritic (senile) plaques (75). The diffuse plaques are Aβ positive but it is not clear whether they are related to AD or part of aging, since they have been found in brains of elderly people that are cognitively intact (76), whereas the neuritic plaques are often found in AD patients. The neuritic plaques are extracellular, contain a dense core of insoluble Aβ40, Aβ42 and Aβ43 and they are surrounded by dystrophic neurites and active microglia (77). Their formation is believed to contribute to neuronal dysfunction and cell death, although the underlying mechanisms are largely unknown (78). In 1992, Hardy and Higgins suggested that the "deposition of Aβ peptide is neurotoxic, causing the formation of neurofibrillary tangles (NFT), vascular damage, cell death and dementia, being the trigger or even the driver of the pathology" (79). This is referred to as the amyloid cascade hypothesis. The evolution of the amyloid deposition was proposed by Braak in a three-stage scheme. Firstly, the frontal and temporal lobes are affected. In the

second stage, the deposition is extended to the hippocampus, and in a third stage it reaches the subcortical regions and cerebellum (80). Aβ peptide not only can accumulate in the brain parenchyma but also around the blood vessels, which is cerebral called amyloid angiopathy (81).

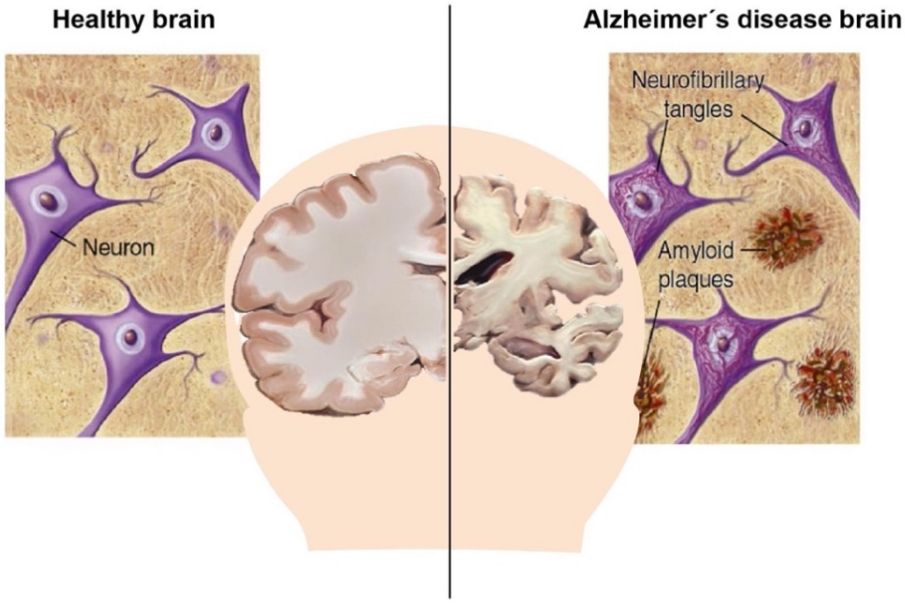

Figure 3. Schematic illustration of a healthy brain (left) and a brain affected by Alzheimer´s disease (right). In the AD brain, the presence of amyloid plaques and tangles of phosphorylated tau lead to atrophy of the brain. Images were adapted from Brain image from Elsevier Sci. Tech connect and BrightFocus Foundation with permission.

Almost at the same time as Aβ was identified, it was found that the microtubule-associated protein tau was present in NFT, the second pathological hallmark of AD (Figure 3) (82, 83). Tau is a cytoskeletal protein that stabilizes microtubules. In AD, tau is hyperphosphorylated and its physiological function is impaired, which causes microtubule disassembly (84), and formation of NFT (85). There is evidence that support that the number of NFTs correlate better with dementia than the amyloid plaque load does (86, 87). However, mutations in tau protein are not found in AD, but they instead cause frontotemporal dementia (88). A number of other phenomena have been implicated in the pathogenesis of AD, including mitochondrial dysfunction (89), oxidative stress (90), inflammation (91, 92) and impaired autophagy (93).

The vast majority of AD cases are late-onset or sporadic and they occur after 65 years (94), while less than 5% of AD cases are early-onset (95), and caused by genetic alterations (43). The first discovered genetic determinant of early-onset familial AD was a point mutation in the APP gene, causing an amino acid residue substitution (Val→Ile) in the C-terminal end of the β-amyloid peptide (Aβ) (96). Early-onset, familial AD cases (79) are overall more aggressive and they are caused by mutations affecting either the cleavage sites for Aβ generation, the Aβ amino acid sequence as such, or the secretase enzymes that cleave out Aβ, in particular the γ-secretase (97). Since the first description of an APP mutation in AD, additional mutations in APP, presenilin (PSEN) 1 and PSEN2 have been found to account for early-onset familial AD

cases. To date, 31 mutations have been reported for APP, 177 for PSEN1 and 14 for PSEN2. (98). Deposition of Aβ into plaques apparently occurs during normal aging, since plaques can be found also in non-AD cases and there is no correlation between the plaque load post-mortem and cognitive decline in AD patients (99, 100). However, most of the described mutations lead to an increase in the ratio of Aβ42/Aβ40 (101). These data imply that Aβ levels, in particular Aβ42, are important for the development of the disease. Late-onset or sporadic AD, found in patients with no apparent family history of dementia is considered to be a heterogeneous and a multifactorial disease that combines several genetic and environmental factors (65). For sporadic late-onset AD, the first susceptibility gene discovered was the epsilon (ε) allele of apolipoprotein E (APOE) gene, on chromosome 19 (102). APOE has three alleles: APOE-ε2, APOE-ε3 and APOE-ε4, ε4 being the one that represents a major susceptibility factor for AD, increasing the risk by fourfold when inherited in one copy and tenfold for two copies (4). APOE is known to be involved in lipid metabolism and transport (103), but also in the clearance of Aβ from the brain (4). Genome-wide association studies (104) have identified several genes implicated in sporadic AD, associated with inflammation (CR1, TREM2) (104-106), vesicular trafficking (PICALM) and cholesterol metabolism (APOE, CLU) (107) as players that affect the susceptibility to develop AD (108).

2.2.2 The amyloid precursor protein

APP belongs to a small gene family that also includes genes encoding the APP-like proteins (APLPs) APLP1 and APLP2. This very well conserved family is found in vertebrates and some invertebrates but not in prokaryotes, plants or yeast, suggesting that APP is linked to the evolution of a nervous system. Although APLPs do not produce Aβ, they undergo sequential processing by different secretases, giving rise to fragments with different functions (109).

APP is an approximately 100 kDa type I single-pass transmembrane protein with a large extracellular domain and a short cytoplasmic fragment (110). APP is found in the secretory pathway, endocytic compartments and at the cell surface (111). The short C-terminal cytoplasmic domain is the most conserved part in the APP family. APP is encoded by a single gene, and differential splicing leads to three different isoforms: APP695, APP751 and APP770. The Aβ sequence is present in APP but not in the APLPs. Processing of APP by α- or β-secretase releases soluble fragments, sAPPα and sAPPβ, respectively, that contribute about 50% of the total APP forms in the brain (112).

APP mRNA is expressed in the nervous system (brain, spinal cord, retina), muscle, immune system, pancreas, kidney, thyroid gland and prostate gland (113). APP and APLP2 proteins are ubiquitously expressed, while APLP1 expression is restricted to the central nervous system (114). In the CNS, APP695 is found in the pre- and post-synaptic compartments of excitatory neurons and GABAergic interneurons, mainly in the hippocampus and cortex (115). APP matures through the secretory pathway (116), and after sorting in the ER and Golgi, APP is transported to the axon (117). When it reaches the cell surface, APP is either cleaved by α-secretase and then γ-secretase, or re-internalized in the clathrin-coated pits into endosomes

containing the proteases BACE1 and γ-secretase (117, 118). The cellular distribution of APP thus influences the way in which it is processed: APP accumulation on the surface favors the non-amyloidogenic pathway, whereas APP localization in intracellular compartments promotes amyloidogenic processing (119).

The biological functions of APP are still not clear, but it can function as a cell surface receptor, controlling signaling and adhesion (120, 121). APP has also been implicated in cell migration (122), cellular trafficking and signaling (123), and neuronal calcium homeostasis (124). Clues about its role have been obtained from the study of organisms with a single APP family member. For example, knock-out of the *Drosophila melanogaster* APP homologue suggests that it is involved in axonal transport and outgrowth (125). APP knock-out mice show reduced body weight, reduced locomotor activity and axonal transport defects (126, 127). Double knockouts for APP + APLP2 or APLP1 + APLP2, result in death after birth (128), suggesting that APLP2 could compensate the loss of APP and APLP1.

2.2.3 APP processing

APP undergoes proteolytic processing as it is sequentially cleaved by α-, β-, and γ-secretases, generating different fragments (129, 130). There are two main processing pathways of APP: the non-amyloidogenic and the amyloidogenic pathway (Figure 4). During the non-amyloidogenic pathway APP is cleaved by α-secretase, a protease that belongs to the ADAM (a disintegrin and metalloprotease) family, including ADAM9, ADAM10 and ADAM17 (131). ADAM10 cleavage, within the Aβ sequence, releases a soluble ectodomain called sAPPα (132) and leaves a membrane bound C-terminal fragment referred to as CTFα or C83. The C83 fragment is either degraded or further cleaved by γ-secretase within the transmembrane domain and then generates a 3 kDa peptide, p3, and an APP intracellular domain (AICD). The γ-secretase is a multiprotein complex consisting of PSEN1 or PSEN2, nicastrin, anterior pharynx defective 1 and presenilin enhancer protein 2 (125, 133). Caspases or caspase-like proteins cleave the AICD into two fragments: Jcasp (the N-terminal part) and C31 (the C-terminal part) (130). The name non-amyloidogenic pathway refers to the fact that α-secretase cleaves within the Aβ domain, thereby preventing the generation of Aβ (125). The non-amyloidogenic pathway can be stimulated by neuronal and synaptic activity, thus reducing the formation of Aβ and liberating APPsα (134). The sAPPα fragment was found to be neuroprotective and important in neurogenesis (135), while AICD has been implicated in negatively modulating neurogenesis (136).

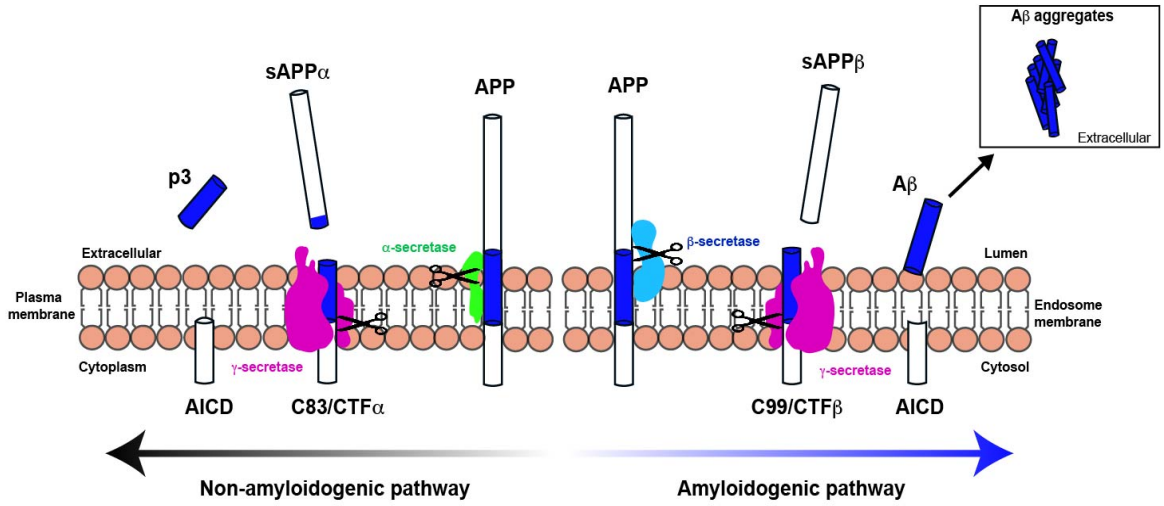

Figure 4. Schematic representation of the two main APP processing pathways. In the non-amyloidogenic pathway APP protein is first cleaved by α-secretase generating C83 and sAPPα fragments, followed by intramembrane proteolysis of C83 by γ-secretase into AICD and p3. In the amyloidogenic pathway APP undergoes sequential processing by β-secretase, generating the C99 and sAPPβ fragments, and γ-secretase leading to the production of AICD and Aβ.

The amyloidogenic pathway is initiated by β-secretase. The major β-secretase is the β-site APP-cleaving enzyme 1, referred as BACE-1, localized mainly in endosomes, lysosomes or trans-Golgi network, and its activity is dependent on an acidic environment (137). BACE-1 cleaves APP at the N-terminal position of Aβ and generates a soluble APP fragment, sAPPβ, which is released into the lumen (129). The remaining membrane bound fragment, referred to as C99 or CTFβ, is a substrate of γ-secretase, which generates a similar AICD that is produced in the non-amyloidogenic pathway (129). Aβ of different length in the range of 34-50 amino acid residues are produced, the 40, 42 and, to some extent, 43 residues peptides being most abundant (125). Studies indicate that BACE1 interacts with APP mainly in endosomes (138) while γ-secretase activity is present on the cell surface and in endosomal compartments (139). It has been shown that neurotoxicity can be exerted by CTFβ *in vitro* (140) and *in vivo* (141).

2.2.4 The Aβ peptide

Aβ is mainly generated in the brain, but is also found outside the brain. The function of Aβ is largely unknown, but it has been ascribed different functions, including cholesterol transporter, signaling molecule, antimicrobial peptide (130) and endogenous antioxidant at nanomolar concentrations (142). It is experimentally complicated to study Aβ due to its disordered conformation and strong tendency to adopt different assembly states (143, 144). Most of the Aβ generated under normal conditions (approximately 90%) is Aβ40, while less than 10% of total generated Aβ is Aβ42 (130). However, the more aggregation-prone Aβ42 and Aβ43 are the main species found in the AD plaques (130, 145-147). Aβ40 is less toxic than Aβ42, it can inhibit Aβ42 oligomerization, and it can promote neurogenesis (148, 149). The more toxic nature of Aβ42 compared to Aβ40 is likely caused by its more pronounced tendency to aggregate and form amyloid fibrils. It is believed that soluble Aβ42 oligomers are the main

neurotoxic species (150, 151), but not all species of Aβ42 are toxic (152) and Aβ42 monomers even show a neuroprotective effects, especially at very low concentrations (153). Under normal conditions, Aβ is continuously generated and eliminated (154). The degradation of Aβ is performed by different proteases, including neprilysin and insulin-degrading enzyme, and by autophagy (155).

2.2.5 The amyloid cascade hypothesis

As stated above, the amyloid cascade hypothesis posits that accumulation of Aβ aggregates is the main causative event in AD, and leads to formation of neurofibrillary tangles, synapse dysfunction, cell loss, and eventually dementia (79, 156). This hypothesis has guided the development of most treatment strategies against AD.

Most evidence supporting this theory are based on the observations that individuals suffering from early-onset AD carry autosomal-dominant mutations that lead to the increased formation of longer and more aggregation-prone forms of Aβ peptides (157). The majority of APP mutations affect the proteolysis of APP in favor of the generation of Aβ42 (158). Interestingly, a rare genetic mutation (A673T) in APP identified in Iceland is protective against AD and cognitive decline, and it has been shown to decrease sAPPβ and Aβ production in cells (159).

However, the amyloid cascade hypothesis has been challenged on several grounds. One argument against the hypothesis is the existence of elderly individuals with amyloid plaques but no symptoms of dementia (75). However, soluble Aβ species, mainly oligomers, are apparently the main toxic species (160, 161) and they show a stronger correlation with cognitive decline than plaque number does (160, 162). As mentioned before, the plaque burden does not correlate with the severity of dementia and the amyloid deposits can be also found in elderly brains of healthy individuals (163). In addition, the large insoluble aggregates, such as Aβ plaques, do not lead to memory impairment in the absence of oligomers in transgenic mice, while a reduction of oligomers lead to cognitive improvement (164). In contrast, in the absence of plaques, the presence of soluble oligomers has a relation with cognitive deficits (165). This is the reason why in the last years more focus has been directed to the oligomers (166-168). Supporting this, there are electrophysiology experiments that have shown that soluble oligomers of Aβ can inhibit hippocampal long-term potentiation (169). Moreover, some reports have shown that oligomers are involved in inducing cell membrane disruption and pores (170), disrupting synaptic function in hippocampal neurons (171), impairment of the ubiquitin-proteasome degradation system (172), mitochondrial dysfunction (173), and apoptosis (174). Although most studies have been focused on extracellular Aβ plaque formation, some suggest that Aβ could start to accumulate inside neurons (175). Another concern is that transgenic mice with pronounced plaque burden do not suffer from a strong neuronal loss (176). But the amyloid cascade hypothesis has received support from the observations that mouse models of Aβ42 deposition suffer from neuroinflammation, synaptic degeneration and cognitive decline (177-180). All these observations have given the community enough support to give the green light for a series of clinical trials to test anti-amyloid drugs, some of which will be described in section 2.2.8.

2.2.6 Kinetics of Aβ fibril formation

The pathways and discrete steps that generate fibrils from monomeric Aβ have been described at a molecular level (Figure 5) (181). Aβ fibril formation is a polymerization mechanism that shows a sigmoidal growth curve. The fibrillation kinetics show a lag phase with little observed fibril growth. In this phase there is a generation of new aggregates at a rate dependent on the concentration of Aβ monomers and independent of the existing fibrils. The lag phase is not a simple waiting step, but it involves a dynamic exchange of monomers and oligomers species, without the presence of any fibril. This initial step is referred to as *primary nucleation*. When a critical concentration of oligomers is reached, the process follows an expansion phase, where the fibrils elongate rapidly at the ends (*elongation*), and at the same time they act as a surface catalyzer for the formation of (toxic) oligomers from monomers in an exponential manner. This step is called *secondary nucleation* (12, 182), and there is evidence that it is the main source of toxic Aβ oligomers (12, 183). These steps depend on both monomers and fibril concentrations (182, 183). Another secondary nucleation process is fragmentation, only dependent on the concentration of fibrils and it happens under shear forces, eg. by agitation, and the smaller pieces increase the overall growth (184). When all the existing oligomers are converted into fibrils, the growth ends. Structures at atomic resolution have been determined for Aβ40 and Aβ42 fibrils, including fibrils from patient brain material, using solid state NMR and cryogenic electron microscopy (185).

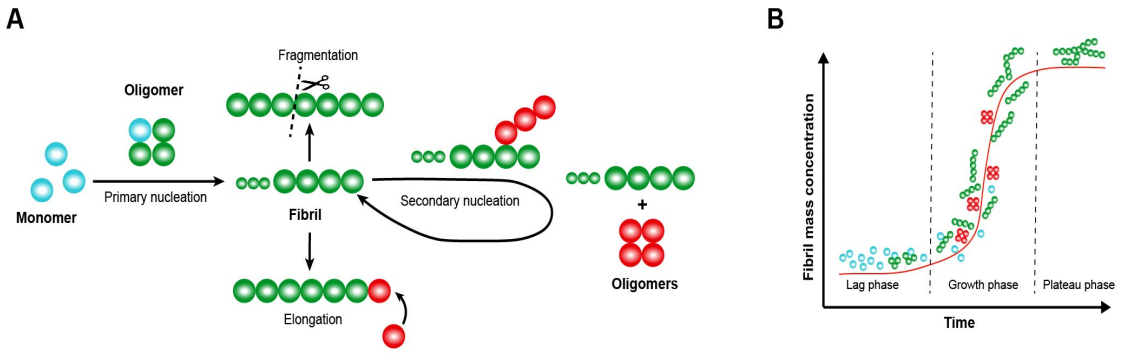

Figure 5. Schematic illustration of the kinetics of Aβ aggregation and fibril formation. (A) Monomers form oligomers during the primary nucleation process and that leads to the formation of fibrils. Fibrils can be formed in a monomer-independent manner, by fragmentation, or in a monomer-dependent manner, by elongation. During the secondary nucleation step, oligomers are formed from the monomers binding at the surface of the fibrils. (B) Schematic example of the kinetics of amyloid fibril formation showing three different phases of the fibril growth: lag phase, growth phase and plateau phase, where different species are present. Panel (A) is adapted from Cohen et al., 2013 (183).

The oligomers species formed during this process adopt different structures, but they can generally be defined as small assemblies of Aβ (186) and as of today, they are considered to be the most toxic and pathogenic form of Aβ. Recently, a designed monomeric form of rh BRICHOS was found to bind to the smallest Aβ oligomers that are competent to catalyze secondary nucleation, and were found to contain eight or less Aβ molecules (187).

2.2.7 The difficulties in finding a therapy for AD

Despite many years of research on AD towards the development of new therapies, there is still no effective way to cure or inhibit the progression of AD. The existing approved drugs on the market are few and they do not cure or stop the progression of the disease, but they affect the symptoms of the patients, although their benefits have been limited (188). There are only four existing drugs approved by the US Food and Drug Administration (FDA) for AD. Three of them (Donepezil, Rivastigmine and Galantamine) are acetylcholinesterase inhibitors (189) and one is a N-methyl-D-aspartate (NMDA) receptor antagonist (Memantine) (190). The long-term efficacy of these drugs is still not clear, and although they do not modify the pathology, they relieve some of the symptoms, improving the quality of life of the patients. A crucial challenge that AD drug development faces is the lack of robust biomarkers of the disease, although the existing CSF and brain imaging biomarkers have provided valuable information (65). Another limitation is the fact that there is a lack of symptoms during the first face (prodromal) of AD and that the patients normally involved in the clinical trials thus have an advanced pathology. Therefore, it is important to consider that the time of treatment could be relevant for the success of the drugs. Nevertheless, there has been a progress in imaging strategies that will help to predict the stage of the disease (65).

The ongoing research is mainly focused on Aβ, based on the amyloid cascade hypothesis. There are several drug candidates for AD treatment in late phases of clinical development and most of them are focused on reducing Aβ production and/or increasing clearance. The strategies include antibodies against Aβ and inhibitors and modulators of β-secretase and γ-secretase, aiming to clear Aβ or slow down the aggregation. A few γ-secretase inhibitors have been tested, as for instance Semagacestat (Eli Lilly & Co.), but failed during phase III clinical trial (191). This was partially due to the adverse effects of γ-secretase inhibitors have on Notch cleavage, an important regulator of neuronal stem cells. A different γ-secretase inhibitor, Avagacestat (Bristol-Myers Squibb) gave serious side effects such as cerebral microbleeds or nonmelanoma skin cancer (192). For this reason, a new generation of γ-secretase inhibitors or modulators was developed (193), such as EVP-0962 (FORUM Pharmaceuticals, Inc.) with little or no effect on Notch proteolysis. However, studies in clinical trial phase II were terminated in 2016 (194). A different approach is to use BACE1 inhibitors (195). Despite all the efforts, many have failed due to low blood brain barrier (BBB) penetration or unacceptable liver toxicity. Currently there are some BACE1 inhibitors in Phase II/III trials, for example AZD3293 (AstraZeneca, PLC, Eli Lilly & Co.) and E2609 (Biogen, Inc., Eisai Co., Ltd.) (65).

Another approach is the use of immunotherapy, both active and passive immunization, through the administration or generation of antibodies to clear Aβ load in the brain. The first active immunotherapies with the vaccine, AN-1792 (Janssen Pharmaceutica, Pfizer, Inc.) failed causing severe meningoencephalitis (196). Another vaccine, CAD106 (Novartis Pharmaceuticals), went into phase III but was recently terminated. Passive immunization ensures more consistent antibody titers and may therefore allow to control the responses. Over the last years, several monoclonal antibodies (mAbs) that bind and reduce Aβ have been

developed (Table 1). Bapinezumab (Pfizer, Inc., Johnson&Johnson Parmaceutical Company, Janssen Pharmaceutica and Elan Pharmaceuticals) was the first mAb targeting the N-terminal part of Aβ ($Aβ_{1-5}$) to be tested in humans. The phase III study was terminated due to a lack of efficacy in AD patients and the appearance of side effects (197). Solanezumab (Eli Lilly & Co.) was developed to target soluble monomers rather than fibrillar Aβ, and it binds to the mid-domain of Aβ ($Aβ_{16-26}$), however, like for Bapinezumab, the studies ended in phase III due to lack of significant results and secondary effects in the patients. Currently there are a few mAbs in phase III clinical trials, including Ganterenumab (Chugai Pharmaceuticals Co., Roche, Ltd.) designed to bind aggregated forms of Aβ, with N-terminal and central epitopes; Aducanumab (Biogen), designed to bind Aβ aggregates including soluble oligomers but not monomers, and BAN2401 (BioArtic Neuroscience AB, Biogen, Inc., Eisai Co., Ltd.) designed to selectively bind and clear soluble Aβ protofibrils (198). Although some promising results have been obtained, most of the AD clinical trials in phase III have failed so far (199).

Table 1. Monoclonal antibodies bind to different epitopes and Aβ conformations. The epitopes, conformations recognized, origin and manufacturers of selected monoclonal antibodies are shown. Dashes indicate absence of information. AA, amino acid. Table adapted from Van Dyck, C.H., 2018 (200).

				Conformations Recognized		
Antibody	**Manufacturer**	**Origin**	**Epitope**	**Monomer**	**Oligomer**	**Fibril**
Bapineuzumab	Pfizer/Janssen Pharma., Inc.	Humanized	AA (1-5)	Yes	Yes	Yes
Solanezumab	Eli Lilly and Company	Humanized	AA (16-26)	Yes	No	No
Gantenerumab	Hoffman-La Roche	Human	AA (3-12), (18-27)	Weak	Yes	Yes
Crenezumab	Genentech, Inc.	Humanized	AA (13-24)	Yes	Yes	Yes
Ponezumab	Pfizer Inc.	Humanized	AA (30-40)	Yes	No	No
BAN2401	BioArtic Neuroscience, AB/Eisai Co., Ltd.	Humanized	Aβ protofibrils	-	-	-
Aducanumab	Biogen, Inc.	Human	AA (3-6)	No	Yes	Yes

The failures of anti-amyloidogenic drugs put in question the amyloid cascade hypothesis, but AD is heterogeneous and there are many factors involved, as for instance the ability of the drugs to cross the BBB. Moreover, the AD animal models available do not reflect the overall complexity of the disease (65).

With this in mind, it seems worthwhile to look at the proteostasis network to find an alternative to prevent Aβ aggregation and toxicity, as a new strategy for treating AD. An advantage of this approach is that it could be applied to several degenerative diseases where protein aggregation

is involved. In this context, the naturally occurring molecular chaperone domain BRICHOS holds potential as a specific amyloid toxicity inhibitor.

2.3 THE BRICHOS CONTAINING PROTEINS AND THE BRICHOS DOMAIN

Sanchez-Pulido et al. identified, in 2002, the BRICHOS domain by multiple sequence alignments of the protein families Bri2, Chondromodulin-I, Tenemodulin, CA11 and surfactant protein C (7). BRICHOS consists of approximately 100 amino acid residues, is observed in several unrelated proteins linked to diseases such as FBD and FDD, respiratory diseases and cancer. The name was given after the protein family members BRI2, CHOndromodulin-I (ChM-I) and proSurfactant protein C (proSP-C). Bri2 and proSP-C are associated with amyloid formation in FBD/FDD and interstitial lung disease, respectively (201). BRICHOS domains from different protein families have very low sequence identities (down to 15% pairwise identities), but their predicted secondary structures are conserved (202), which suggests that BRICHOS domains may perform similar functions. The proteins containing a BRICHOS domain are type II transmembrane (TM) proteins (7, 202, 203), i.e. the N-terminal part is located in the cytosol. They have a common architecture consisting of four regions: an N-terminal cytosolic part, a hydrophobic TM domain followed by a linker region, the BRICHOS domain, and a C-terminal region (Figure 6). The only exception is proSP-C, which ends by the BRICHOS domain (7, 203). All BRICHOS containing proteins harbor a segment with high β-sheet propensity; located in the C-terminal part, except in proSP-C where the TM region has a very high β-sheet propensity (203). There are only three amino acid residues that are strictly conserved in all BRICHOS domains, two cysteines (Cys) and one aspartic acid (Asp) (202). So far only the structure of proSP-C BRICHOS has been experimentally resolved and it shows a five-stranded β-sheet with one α-helix on each side (Figure 6C), and the two conserved Cys form an intramolecular disulfide bridge (201). Homology models for BRICHOS domains from different proproteins have shown that physicochemical properties of one face of the β-sheet (face A), containing mainly hydrophobic residues in proSP-C, are similar to the properties of the β-sheet prone segments of the corresponding proproteins, i.e. the presumed BRICHOS substrates (203). The work in this thesis has been mainly focused on the BRICHOS domain from Bri2 and proSP-C which therefore will be reviewed in a more detailed manner.

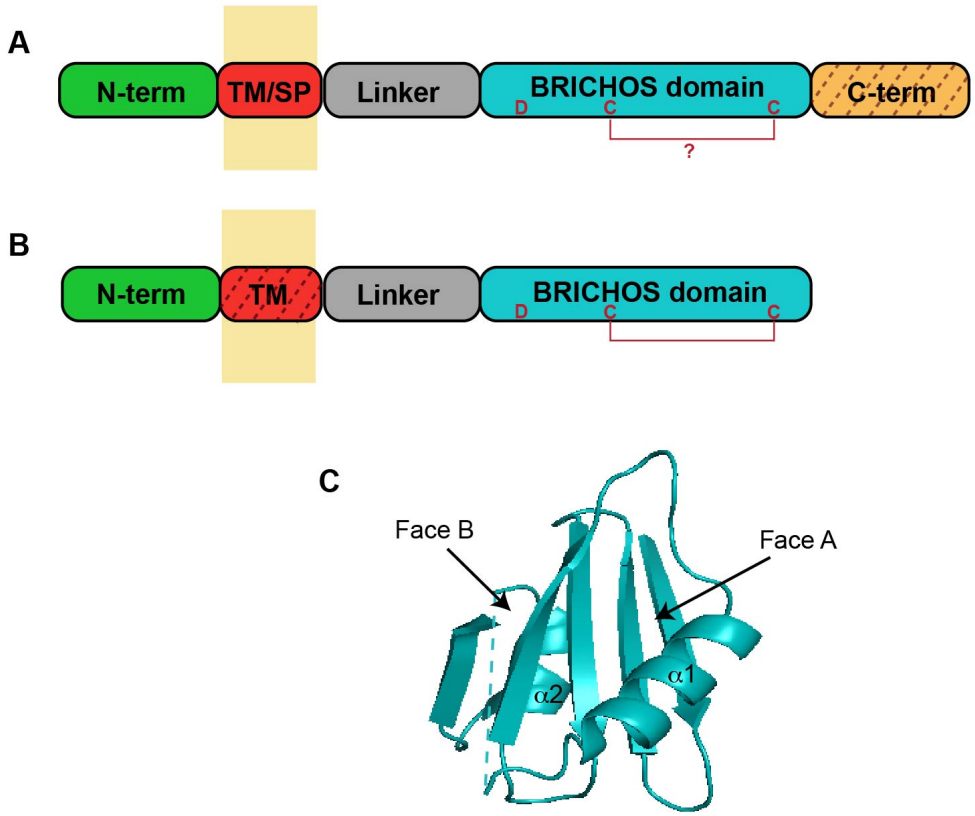

Figure 6. Schematic overview of the architecture of all BRICHOS domain containing proteins (A), except for proSP-C, represented in (B). The N-terminal region is highlighted in green, the TM region or signal peptide (SP) in red, the linker is shown in grey, the BRICHOS domain in blue and the C-terminal region in dark yellow. The aggregation prone regions in proSP-C (B) and other BRICHOS proteins (A) are marked with dashed lines. The BRICHOS domain region contains two conserved Cys (C) and one Asp (D), and the intramolecular disulfide bond between the two C is indicated. (C) Three-dimensional structure of BRICHOS domain of proSP-C (PDB: 2YAD), showing Face A and Face B of the β-sheet.

2.3.1 Prosurfactant protein C

Lung surfactant is a thin film composed of phospholipids and proteins that covers the alveolar aqueous lining. It has two important roles: it reduces the alveolar surface tension thereby preventing the lungs from collapse at end of expiration, and it plays a role in innate immunity protecting the lungs from pathogens (204). Lack of sufficient amounts of surfactant in prematurely born infants is associated with respiratory distress syndrome (RDS). Surfactant´s ability to reduce surface tension requires two hydrophobic proteins, surfactant protein B (SP-B) and SP-C (205-207).

ProSP-C is exclusively expressed in the secretory pathway of the alveolar type II epithelial cells as a 21 kDa integral membrane precursor protein with 197 amino-acid residues (204). SP-C is secreted, together with SP-B, into the alveolar space along with phospholipids (206). ProSP-C is proteolytically processed to mature 35-residue SP-C along the secretary pathway (208, 209). ProSP-C is first cleaved in the Golgi complex whereby a 30 residue C-terminal segment is removed. Then, a part of the N-terminal region is removed and the remaining segment is transferred to the multivesicular bodies, and finally a 9-residue N-terminal segment

is removed, liberating mature SP-C (204). SP-C is folded into a highly regular α-helix from residues 9-34, whereas the N-terminal octapeptide segment and the C-terminal segment are disordered (208, 210). Mature SP-C is one of the most hydrophobic proteins known and its central hydrophobic domain is comprised by a region rich in isoleucines and valines (211), that are known to have high propensity to amyloid formation (58, 212, 213). When the BRICHOS domain or the linker region of proSP-C are mutated, the TM part that corresponds to mature SP-C forms amyloid-like fibrils, leading to interstitial lung disease (ILD) (201, 204). From the pronounced amyloidogenicity of SP-C, it was hypothesized that the BRICHOS domain of proSP-C is required to prevent amyloid formation (11, 214). This is supported by the finding that BRICHOS dysfunction leads to amyloid disease already in childhood, which has not been described for any other amyloid disease and thus further points to the pronounced amyloid potency of SP-C *in vivo* (201).

2.3.2 Bri2

Full length Bri2 protein is a 266 amino acid residue long (30 kDa) type II TM protein that is encoded by the integral membrane protein 2B (*ITM2B*) gene, located on chromosome 13 (7). Bri2 is ubiquitously expressed at high levels in the brain, especially in hippocampal pyramidal neurons in the CA3 and CA4 regions (215, 216), cerebellum and cortex, but also in peripheral tissues like placenta, kidney and pancreas (217). Full-length Bri2 consists of an N-terminal cytosolic part (residues 1-54), a TM region (residues 55-75), a linker (residues 76-130), a BRICHOS domain (residues 130-231) and a C-terminal region (residues 232-266) (7, 218). Bri2 undergoes proteolytic processing during its transfer through the secretory pathway (Figure 7), first by furin, which releases a 23-residues peptide (Bri23) from the C-terminal region (219), which is capable of inhibiting Aβ aggregation (220). Other proprotein convertases than furin are able to process Bri2, releasing C-terminal peptides (221). The Bri2 BRICHOS domain can be released by cleavage by the α-secretase ADAM10 and it is then secreted into the extracellular space (222). The exact site where APP is processed by ADAM10 has not been identified and data suggest that the cleavage is not sequence-specific, but rather that the enzyme cleaves at a certain distance from the plasma membrane (223). After this, the remaining membrane-associated Bri2 N-terminal fragment undergoes intramembrane proteolysis mediated by signal peptide peptidase-like proteases, (SPPL), SPPL2a and 2b. This releases a Bri2 intracellular domain (ICD) into the cytosol (222).

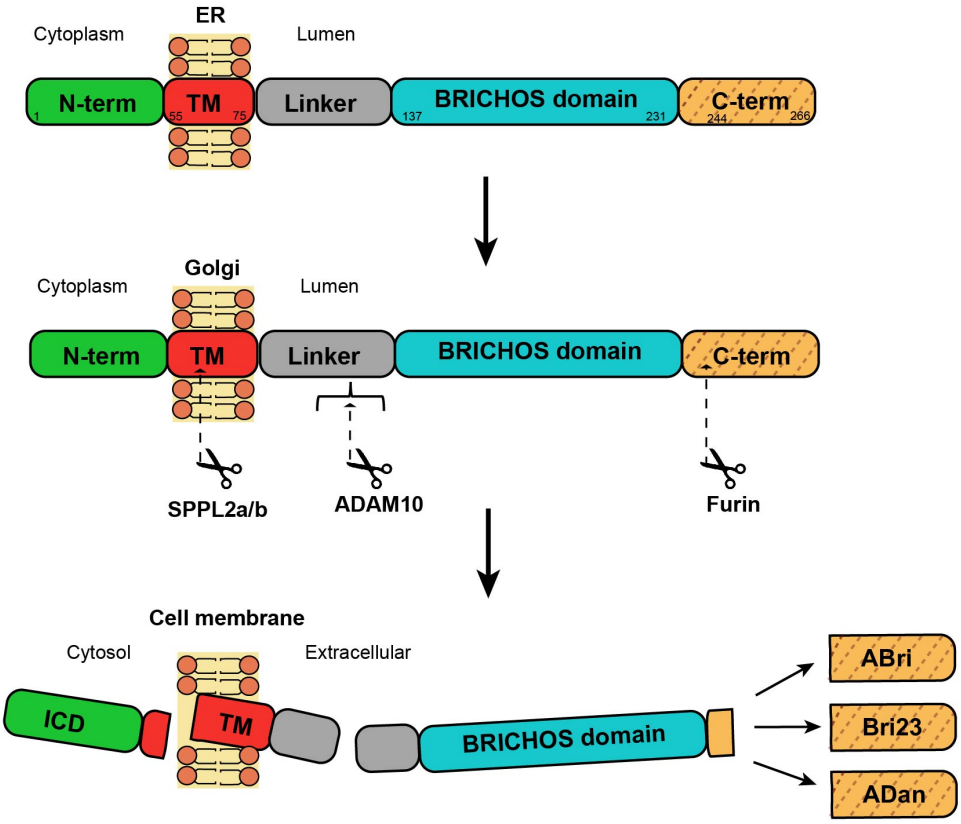

Figure 7. Schematic representation of Bri2 protein and its processing. Bri2 proteins consists of an N-terminal region shown in green, a TM region in red, a linker in grey, the BRICHOS domain in blue and C-terminal domain in dark yellow. Bri2 protein is processed in the secretory pathway first by furin in the C-terminal region, followed by ADAM10 in the linker and by SPPL2a or SPPL2b in the transmembrane region.

Mutations in the *ITM2B* gene give rise to the release of extended C-terminal peptides, ABri or ADan that deposit in the CNS leading to dementia, FDD and FBD, and both diseases share neuropathological similarities with AD (224). FBD is the result of a mutation in the stop codon of the Bri2 gene that leads to 34 amino acids long ABri peptide, instead of the 23-residue long Bri23 peptide (225). On the other hand, the FDD mutation results in a 10-nucleotide duplication prior to the frame stop codon, likewise extending the resulting peptide by 11 residues. Even though ABri and ADan are the same length, their sequences differ. Two hypotheses have been suggested to explain the mechanisms underlying these two rare diseases: the first hypothesis focuses on the gain of a toxic function of the extended C-terminal peptides, resembling Aβ, and the second hypothesis proposes that the mutations in *ITM2B* contributes to a loss of function of Bri2 protein, which is degraded and as a consequence, leads to deregulation of APP processing (225). This last hypothesis is supported by results showing that Bri2 physically restricts APP from the secretases that are responsible of APP processing, thereby modulating Aβ generation and reducing the amyloid plaque load (226-229). This suggests an association of Bri2 with APP metabolism. Moreover, it has been observed, by co-precipitation studies, that Bri2 interacts with BACE1 in cell cultures, inducing its degradation (230). A study by Kim and co-workers showed that transgenic mice that overexpress Bri2 together with Aβ42 developed amyloid pathology but had less deposition compared to transgenic APP mice, and importantly

they lacked the expected cognitive impairment (231). Taken together, these studies suggest a relevant role of Bri2 in AD pathogenesis and also support that the BRICHOS domain has a physiological role in preventing Aβ toxicity.

2.3.3 The BRICHOS domain in AD and Aβ context

From the apparent involvement of proSP-C BRICHOS in correct folding to prevent SP-C amyloid formation (201, 232), the idea was born that BRICHOS domains might protect against formation of amyloid fibrils more generally (9, 10, 14, 15, 38). Indeed, both recombinant human (rh) proSP-C and Bri2 BRICHOS could inhibit fibril formation of Aβ40 and Aβ42 *in vitro*, below stoichiometric ratios (12, 13, 16). Different BRICHOS domains have different effects on Aβ42 aggregation; proSP-C BRICHOS binds to the surface of Aβ42 fibrils but not to monomers, and blocks secondary nucleation, while rh Bri2 BRICHOS inhibits both secondary nucleation and elongation pathways (12, 13, 18, 233). Data obtained from surface plasmon resonance and immune electron microscopy support that proSP-C BRICHOS binds to fibrillar but not monomeric species of Aβ42 (13, 187). Rh Bri2 BRICHOS domains form assemblies with distinct activities: monomeric species are most effective in inhibiting Aβ42 neuronal network toxicity, dimers efficiently target Aβ42 fibril formation, and the high molecular weight assemblies are most efficient in inhibiting non-fibrillar protein aggregation, acting as a traditional chaperone (233). This is not the case for rh proSP-C BRICHOS, that does not possess a general chaperone activity and is only active against amyloid fibril formation (18, 201, 234).

In vivo studies of proSP-C and Bri2 BRICHOS performed in a *Drosophila melanogaster* transgenic model of AD, in which Aβ42 and proSP-C or Bri2 BRICHOS are overexpressed, showed delayed Aβ42 fibril formation, improvement of locomotor activity and increase of lifespan compared to the flies expressing Aβ42 only (17, 18). These results support the idea of using BRICHOS as a potential therapeutic molecule against AD. Additionally, adding rh Bri2 or proSP-C BRICHOS domains to hippocampal mouse brain slices reduces neuronal network toxicity caused by Aβ42 (13, 14, 18, 233). Studies in patients in early stages of AD found that Bri2 levels are increased up to 3-fold in the hippocampus, suggesting that disturbed Bri2 metabolism is associated with AD (6).

2.4 ANIMAL MODELS TO STUDY AD

The use of animal models has been crucial to provide researchers with important knowledge about AD. The discovery of genes that affect familial forms of AD allowed scientists to create models of the disease, especially in mice. The Alzforum website (www.alzforum.org) has referenced up to 205 different models in their database, as of April 2021. Many organisms, from mice to worms (*Caenorhabditis elegans*) or fruit flies (*Drosophila melanogaster*), have been used to mimic aspects of AD (235). Some animals present lesions in their brain like the ones found in AD. For instance, dogs can develop diffuse plaques in the cortex (236), and cats (237) and sheep (238) have amyloid plaques but not neurofibrillary tangles. It is probable that non-human primates are the closest to human with senile plaques in the cortex (239, 240),

although it is not known if they develop AD-like phenotypes, and these animals are not commonly used to study AD. Currently there is a project (IMPRiD) to mimic the progression of the disease in macaques by injecting them with human brain tissue. In addition, RIKEN Centre for Brain Science in Japan, has created a marmoset model of AD, that can develop both amyloid plaques and tau tangles, with mutations in *PSEN1* gene (published on bioRXiv). But for now, there are many ethical controversies, and the high costs of non-human primates also constitutes a disadvantage.

The most frequently used specie of AD animal models is the mouse, and many models are based on the overexpression of mutant human APP or Aβ42 that result in the accumulation of Aβ in the brain. The nature and number of the plaques and the age at which the deposition starts depend on the mutations and therefore varies between different models. For instance, two widely used models are APP23 and CRND8. The first model contains the Swedish *APP* mutation (K670/M67L) and develops amyloid plaques in the brain parenchyma starting at 6 months age (241). The second model, CRND8, combines two mutations in the *APP* gene which results in a stronger pathology with amyloid plaques at 3 months age (242). However, these models based on APP overexpression can give artefactual phenotypes because of a foreign gene being inserted at unknown location in the genome, non-natural promoters of gene transcription, and that high production of APP and non-Aβ processing products can give non-physiological effects.

2.4.1 *App knock-in* mice

Because of the drawbacks associated with models based on transgenic over expression, in 2014 Saito and co-workers at RIKEN generated *App* knock-in mouse models in which endogenous mouse *APP* gene is mutated, so that human Aβ42 is over produced, due to the insertion of the Swedish and the Beyreuther/Iberian mutations (App^{NL-F}), or an additional Arctic mutation (App^{NL-G-F}). However, other APP processing products are generated at normal levels (243). The Swedish mutation increases the β site cleavage of APP and thus produces an augmentation of the amounts of total Aβ40 and Aβ42 (244, 245), the Beyreuther/Iberian mutation affects the cleavage of γ-secretase so that increased Aβ42/Aβ40 ratio is obtained, and the Arctic mutation increases the aggregation propensity and promotes a more aggressive Aβ pathology (Figure 8) (246). App^{NL-F} mice show Aβ accumulation from 6 months, and memory impairment is observed at 18 months age (243). The App^{NL-G-F} model shows a greater and more rapid Aβ deposition, both in cortical and subcortical areas, and starts at 3-4 months age and cognitive deficits are observed already at 6 months (243, 244). A more recent report has shown that App^{NL-F} mice present detectable behavioral deficits already at 10 months, although more modest compared to App^{NL-G-F} mice (180). The App^{NL-F} and App^{NL-G-F} models are thus relevant for studies on the downstream consequences of Aβ pathology and both models show Aβ pathology, neuroinflammation, and memory impairment in an age-dependent manner (243). In addition, the memory impairment observed in these *App* knock-in mice reflect the pathological changes seen in AD patients (180, 243, 247). The *App* knock-in mice, however, do not develop tau pathology or neurodegeneration (244).

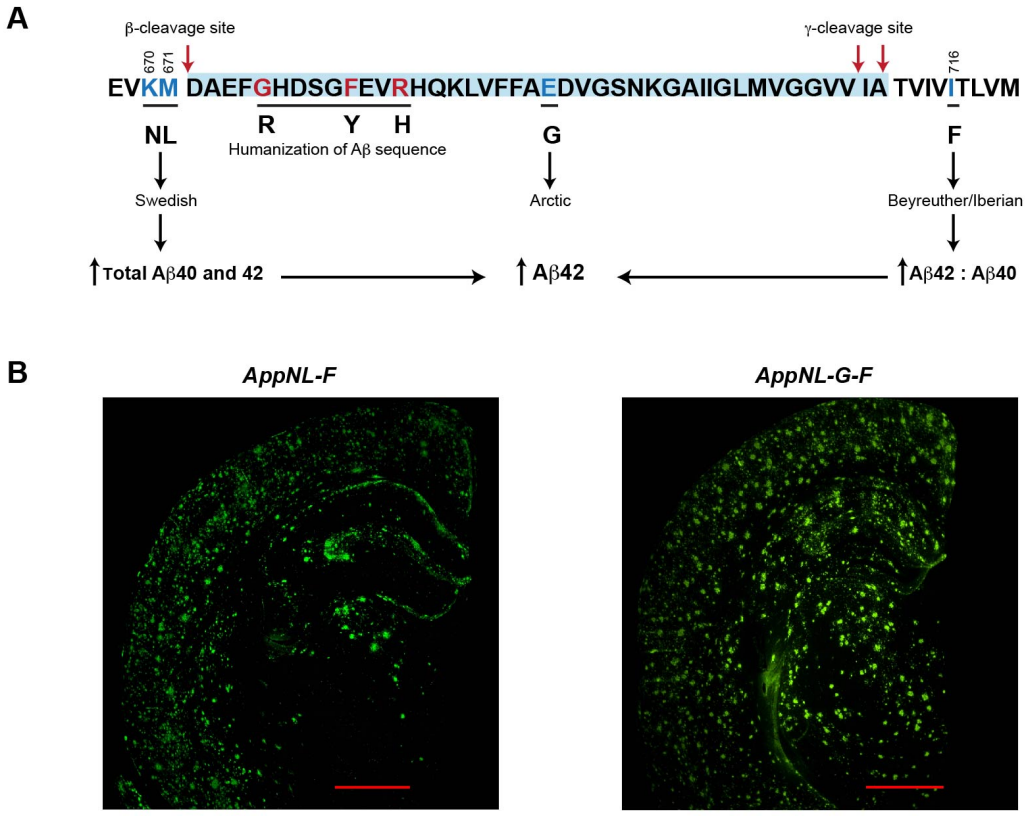

Figure 8. *APP* sequence showing the design of App knock-in mice and the mutations introduced in both models (A) and images of Aβ plaques in the brain of 23 months old *App*$^{NL-F}$ and 7 months old *App*$^{NL-G-F}$ mice (B). (A) *APP* sequence scheme with Aβ sequence higlighted in blue color, adapted with permission from (244). (B) Representative images of *App*$^{NL-F}$ and *App*$^{NL-G-F}$ mouse brains stained for amyloid plaques with 82E1Aβ antibody. Scale bars = 1 mm. Image credit to Dr. Shaffi Manchanda.

These models have been useful to understand the complexity of AD-like symptoms even though they do not develop proper AD. However, it is important to keep in mind that they may still have poor translational value, as the structure and biology of an animal brain differ from humans.

2.5 THE BLOOD-BRAIN BARRIER (BBB) AND ITS TRANSPORT SYSTEMS

The BBB, located at the endothelial cells of the brain capillaries, and the blood-cerebrospinal fluid barrier (BCSFB) in the choroid plexus are physical barriers that separate the CNS from the periphery (248). The BBB performs a dual function: it acts as a diffusion barrier that impedes the passage of most compounds from the blood to the brain, and also it provides essential nutrient supply; both functions help to maintain the brain homeostasis. The BBB is composed of endothelial cells, astrocytes, and pericytes. The BBB endothelial cells are different from the ones in the rest of the body. These endothelial cells form a wall around the vessels, and they are connected by intercellular tight junctions that limit and control the paracellular flux of molecules and help in keeping neurotoxic-plasma derived components and pathogens out of the central nervous system (249, 250). The astrocytes and pericytes provide support and regulation to ensure the maintenance of the tight junctions and sometimes, act as a second barrier. The pericytes appear to have a role in angiogenesis, structural integrity and

differentiation of the vessels (249). Together they form the so-called neurovascular unit (Figure 9).

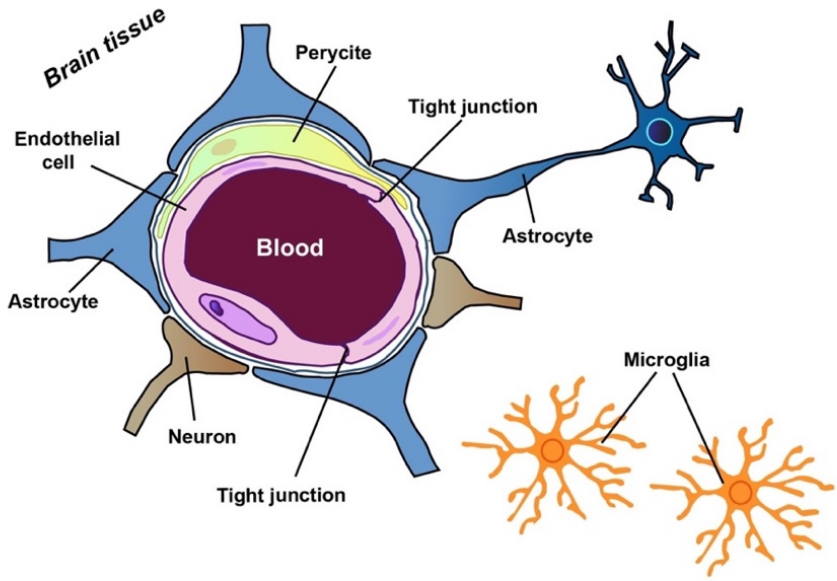

Figure 9. Diagram of the neurovascular unit. The BBB is composed of endothelial cells connected to each other by tight junctions is covered with pericytes and surrounded by neurons, astrocytes and microglia.

The brain consumes a high amount of energy for its normal functioning; with only a mass that constitutes 2% of the whole body, it consumes around 20% of the total energy (251). The BBB presents specialized transport systems that allow nutrients and metabolites into the brain, but also transports waste molecules from the brain to the blood (Figure 10) (250). The endothelium allows free passage over the BBB through paracellular diffusion, by which small molecules (< 400 Da) and lipophilic substances such as oxygen and carbon dioxide (252, 253) can pass the tight junctions. A different transport mechanism is transcellular diffusion, that allows the passage of lipid-soluble molecules by penetrating the endothelial cells (254). Some other molecules like glucose or amino acids are actively transported by selective membrane bound-carrier systems normally present in both the luminal and abluminal membranes of the brain endothelia (254). Some larger molecules, like peptides and proteins such as insulin, transferrin and leptin, cross the BBB by receptor-mediated transcytosis (249, 255-257). During receptor-mediated endocytosis, the protein ligand binds to a specific receptor in the luminal membrane, inducing invaginations, which trigger the formation of endocytic vesicles that transfer the protein ligand into the cell. The vesicles containing the ligand are packed into export vesicles directed to exocytosis in the abluminal membrane, which results in transport of peptides or proteins across the BBB (254). Adsorptive transcytosis is used by cationic proteins, which does not involve specific receptors; and finally efflux transport systems are used to remove substances from the brain or CSF (254). Among the transporters implicated in the efflux, the multidrug resistance transporter P-glycoprotein is best characterized (258, 259).

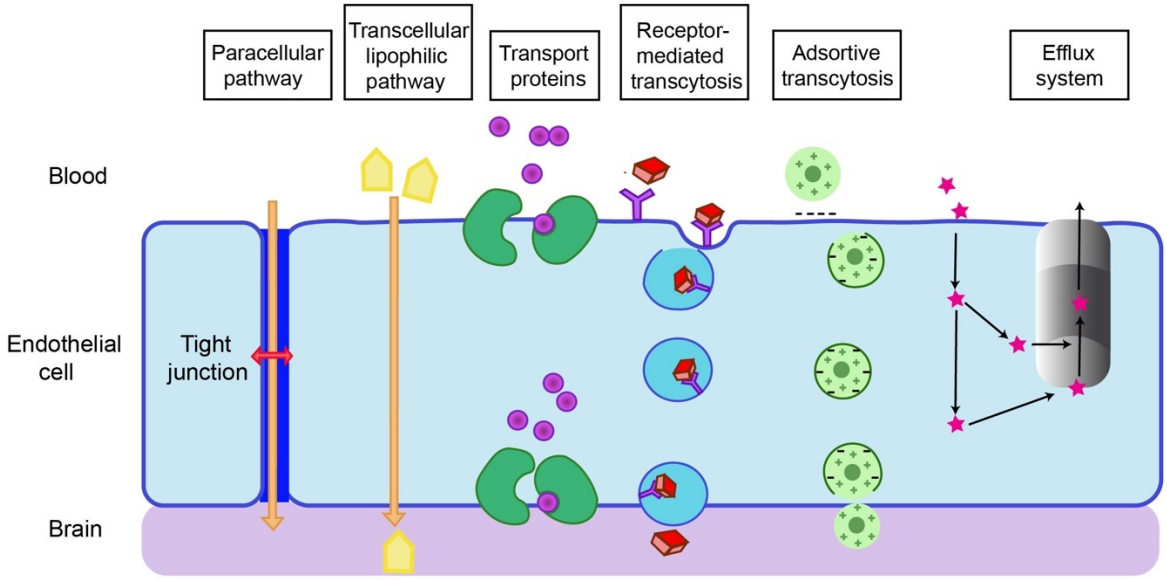

Figure 10. Schematic representation of the different BBB transport mechanisms.

In many CNS pathologies, the BBB is altered, and these changes can include increased leakiness of tight junctions, extravasation of plasma proteins via paracellular or transcellular routes and altered expression of drugs transporters (260). About 40% of patients with AD suffer some form of vascular cognitive impairments (261). In AD, it has been shown by magnetic resonance imaging (MRI) that there is a breakdown of the BBB in the hippocampus (262), leakage and perivascular accumulation of blood-derived proteins, endothelial degeneration, and loss of BBB tight junctions (263). These findings have been supported by research done in APP mutant mouse models harboring the Swedish APP mutation (KM670/671NL), demonstrating BBB leakage, degeneration and loss of brain capillary pericytes, endothelial cells and loss of tight junction proteins (264-266). Additionally, this mouse model has an abnormal expression of BBB transporters and receptors, as for instance a decrease in the levels of low-density lipoprotein receptor-related protein 1, implicated in the clearance of Aβ from the brain to the periphery (267). Additional research work performed on *APP* transgenic mice to study the implication of the BBB breakdown and AD pathology suggest that the degeneration of the BBB occurs before Aβ deposition and that it might be a consequence of toxics effects in the vasculature (268).

2.5.1 Approaches to improve drug delivery to the brain

The success of the treatments for CNS disorders such as AD requires the development of rational strategies to deliver drugs at appropriate concentrations across the BBB. For a drug to cross the BBB passively, the molecule mass should be under a 400 to 500 Da threshold and it should have high lipid solubility (269). There are examples of proteins that have been observed to cross the BBB, i.e. erythropoietin (34 kDa), and serum proteins (270, 271). Moreover, anti-Aβ antibodies of about 150 kDa are able to enter into the brain, bind to amyloid plaques, and cause a reduction in plaque burden in AD mouse models (272) and AD patients (273). Some

technologies to penetrate the BBB and enhance the delivery of drugs have been developed (254):

- Pharmacological approaches: The strategy consists of lipidation and chemical modification of drugs, eg. liposomes or micelles are used, to improve their ability to diffuse across the BBB. Lipid- and polymer-based nanoparticles have also been used to improve the ability to target the therapeutic site (274). The chemical modification of active drugs allows them to increase their diffusion. However, it could produce side effects, as well as decrease the activity of the drug, eg. by increasing their interaction with plasma components (275-277).

- Physiological approaches: This idea is based on the use of the natural receptor-mediated transport system in the brain and consists of the modification of therapeutic molecules by conjugating them to ligands, antibodies or chimeric peptides that bind receptors expressed in the BBB, i.e. the transferrin, leptin or insulin receptors (278-280). Disadvantages are the high cost of the design of the new drugs as well as the impact that the modifications could have on activity.

- Intranasal drug delivery: It is based on the transport into the CNS through olfactory neuronal pathways, and it allows a delivery of the drugs over the BBB within minutes. This approach seems to be more efficient than intravenous administration for some drugs. The drawbacks are low efficiency for drugs with higher molecular weights and the variabilities of the absorption in the nasal mucosa (281).

- Invasive approaches: Convection-enhanced delivery, intra-cerebro-ventricular infusion, intracerebral injection, polymer or microchip systems which directly release therapeutics after implantation in the CNS, and disruption of the BBB by using hyperosmotic or biochemical methods such as bradykinin analogs or mannitol are examples of invasive techniques (282, 283). However, the invasive natures, the need of multiple injections to be clinically feasible and the absence of control of the diffusion of the drug are potential disadvantages (284).

2.6 FOCUSED ULTRASOUND (FUS)

It has been shown that the BBB could be transiently opened by FUS, in combination with polydisperse microbubbles (MBs) (285, 286). FUS in combination with intravenously injected MBs efficiently opens the BBB in a non-invasive, transient and reversible way (285, 287, 288). The acoustic pressure increases as the bubble size decreases (289), and the bubble-size is important to achieve BBB opening (290) and diminish the risk of tissue damage (291). The spot where the opening occurs can be visualized by magnetic resonance imaging (MRI), as MRI-contrast agents will only cross the BBB at the site of the BBB opening (292). Another advantage is that this method does not need surgery which makes it suitable for repeated treatments. FUS has been used in animal models to study diseases that include Parkinson´s

disease, brain tumors and AD (293), in which antibodies (294), nanoparticles(295), viruses (296) and even stem cells (297) have been successfully delivered.

The main hypothesis regarding the mechanisms of BBB disruption is that when FUS is applied, the MBs cavitate locally and temporally due to the FUS waves and they exert a force on the capillary walls causing the tight junctions in the BBB to loosen. Another hypothesis is that FUS temporally opens the membrane of vacuoles present in the cytoplasm of cells, allowing drugs to enter the interstitial space (298). Thus, using FUS we enhance at least four ways of transport systems across the BBB: endocytosis, transcytosis, paracellular transport and through the channel formation or fenestration in the cytoplasm of the endothelium (299). The predominant route is probably between the endothelial cells (paracellular way), during transient opening of the tight junctions in the capillaries after sonication (290). The opening is not only restricted to capillaries or venules; it has been shown that the transport also occurs in arterioles (300). The precise effect of the FUS on mechanisms of BBB transporters remain to be determined.

BBB permeability is normally a consequence of a brain pathology, eg. cerebral ischemia (260) and even a transient and localized BBB opening could have a devastating consequence for the brain. However, there is evidence that FUS with MBs allows a transient BBB opening with no acute neuronal damage (287). Recent studies have shown the safety and feasibility of this method as a potential treatment in AD patients (293).

2.6.1 FUS as a therapy for AD

Several preclinical studies have been conducted in mouse models of AD (301-304) where it is observed that repeated ultrasound treatments could reduce the deposition of Aβ and decrease the amyloid plaque load. In 2018 the first phase I clinical trial to evaluate the safety and feasibility of the FUS in combination with commercially approved MBs in five AD patients with mild-to-moderate stage of the disease was published (293). The targeted area was the frontal lobe, where the patients had amyloid deposition, and the opening and closure (24 hours later) of the BBB was monitored via MRI. In this study, FUS in combination with MBs was proved to be a safe and efficient methodology to induced BBB opening in humans. A later study aiming to understand the feasibility, safety, efficacy and effects of repeated FUS treatments have been conducted in human patients (305). The preclinical results have demonstrated that application of FUS for 3 months is not only safe but also improves memory performance and language in the treated patients. In all, FUS seems promising but further studies to understand the mechanisms of action are needed.

3 AIMS OF THE THESIS

"Above all, don't fear difficult moments. The best comes from them." -Rita Levi-Montalcini

Great efforts are put on AD research and the development of new drugs to resolve and treat the disease, but no successful treatment has been found until date. The *in vitro* mechanisms of rh BRICHOS domains make it a potential candidate against AD; however more *in vivo* experiments are needed. The **overall aim** of this thesis is to provide new insights on the use of rh BRICHOS in animal mouse models as a future strategy for AD.

More specific aims for each study were the following:

⇒ **Paper I**. Our focus was to investigate the blood-brain and blood-cerebrospinal fluid passage as well as serum half-lives of intravenously administered rh Bri2 BRICHOS-AU1 and rh proSP-C BRICHOS domains.

⇒ **Paper II**. In this study our goal was to increase the passage over the blood-brain barrier of rh Bri2 BRICHOS-AU1 and rh proSP-C BRICHOS domains using focused ultrasound in combination with microbubbles and investigate delivery into the brain.

⇒ **Paper III**. Based on Paper II, our focus was to decipher whether lipid microbubbles without focused ultrasound facilitate the passage of rh wt Bri2 BRICHOS-AU1 and rh Bri2 BRICHOS-mCherry domains into the brain.

⇒ **Paper IV**. Here, using the knowledge obtained from Paper I on the delivery of rh Bri2 BRICHOS-AU1 domains, we aimed to study the treatments effects of rh Bri2 BRICHOS R221E monomers, designed to target the neurotoxicity associated with Aβ aggregation in mouse models with AD pathology.

4 MATERIALS AND METHODS

"Though this be madness, yet there is method in't." -William Shakespeare

"If we knew what it was we were doing, it would not be called research, would it?" -Albert Einstein

In this section the ethical considerations and the most relevant *in vitro* and *in vivo* techniques used in the different studies are briefly described. More detailed explanations of all methods are found in **papers I-IV**.

4.1 ETHICAL CONSIDERATIONS

The use of experimental animals can be justified by the aim to improve human health, as long as the procedures are carried out with minimal suffering for the animals. However, the importance of the experiment is partially subjective, and it is complicated to know to what extent the animal experiences pain or other types of discomfort during the experiments that we conduct.

The research using animal models must be performed under the supervision and approval of ethical committees. All the studies presented in this thesis were performed according to the principles stated in the Helsinki Declaration. The studies conducted in **papers I**, **III** and **IV** were approved by the regional ethical committees for animal research in Stockholm south - ethical permits S6-15 and dnr 03049 and by the Linköping´s animal ethical board (ID855). The animal procedures conducted at Columbia University and presented in **papers II** and **III** followed the guidelines of Columbia University Institutional Animal Care and Use committee.

4.2 PRODUCTION OF RECOMBINANT PROTEINS FOR ANIMAL EXPERIMENTS

There is a great number of proteins used as therapeutic agents and one of the major problems as regards of safety and efficacy is their potential adverse effects, e.g. immunological responses. An immune response could be due to different factors: from disease-related factors as genetics or age to product-related factors such as impurities or protein aggregation. In this work we have not analyzed the immunogenicity of proteins, but as we used recombinantly produced proteins from bacteria, they had to be essentially free of contaminants. One of the most frequent contaminations to avoid prior administration into animals and humans is the presence of endotoxins -lipopolysaccharides (LPS)- derived from the outer cell membranes of gram-negative bacteria. These LPS can not only affect the functionality of proteins but also contribute to adverse effects, thus reducing or eliminating them is essential. For that purpose, in this work we have developed a laboratory-scale purification protocol that generate pure proteins.

The rh Bri2 BRICHOS proteins in these studies (**papers I-IV**) were recombinantly produced from a bacterial host, *Escherichia coli*, fused to a spider silk protein derived solubility tag, NT* (13.8 kDa), that helps to maintain the solubility of the proteins (306-308). During the first step of this process, the proteins are extracted by breaking the cells using mechanical disruption (sonication). Thereafter, the proteins are separated from insoluble contaminants by low-speed centrifugation. We can capture the His_6 affinity tagged proteins of interest by a simple protocol using immobilized metal affinity chromatography (Ni^{2+} charged IMAC column), and the His tag is later removed from the recombinant protein by enzymatic digestion, following a second application of the sample to the IMAC. This method largely reduces the number of contaminants, since both impurities that bind and do not bind to IMAC resin will be removed. The degree of protein purity after IMAC purification is often high (about 95%) in the case of highly expressed proteins, as is the case for rh Bri2 BRICHOS. A higher degree of purity can be achieved by further purification using other chromatographic methods as size exclusion chromatography (SEC), hydrophobic interaction or ion exchange chromatographies, as well as endotoxin removal resins with high affinity for LPS, and some of those were applied to produce recombinant proteins in **papers I-IV**.

4.2.1 Isolation of the most anti-neurotoxic species: rh Bri2 BRICHOS monomers

The inhibition of generation of the Aβ42 oligomers that cause neurotoxicity was our main strategy in **paper IV**. Previous data showed that the most potent quaternary structure of Bri2 BRICHOS in preventing neurotoxicity is the monomeric form (233). The isolation of a pure rh Bri2 BRICHOS R221E monomer (19) was achieved by SEC, that separates molecules by different sizes while the sample pass through a column packed with an inert resin composed of a porous matrix of spherical beads made of dextran polymers (Sephadex), agarose (Sepharose) or polyacrylamide (Sephacryl). Since larger molecules cannot enter some pores, they elute faster, which means that the elution time of the proteins is inversely proportional to their shape and size (hydrodynamic volume).

4.3 THE MOUSE MODELS TO STUDY ALZHEIMER´S DISEASE

To investigate the potential treatment effects of rh Bri2 BRICHOS in AD, experimental preclinical studies presented in this doctoral these were carried out in two animal models of this disease. In **paper IV** we have used humanized knock-in mouse models in which the endogenous mouse APP gene is mutated, so that the human Aβ42 is over produced.

4.3.1 App^{NL-F} and App^{NL-G-F} mice

The experimental studies conducted in **paper IV** were carried out in two *App* knock-in mouse models of AD that harbor double mutations (Swedish (KM679/671NL) and Beyreuther/Iberian (I716F) mutations, App^{NL-F}) and triple mutations (Swedish, Beyreuther/Iberian and Artic (E693G) mutations, App^{NL-G-F}) (Figures 8 and 11).

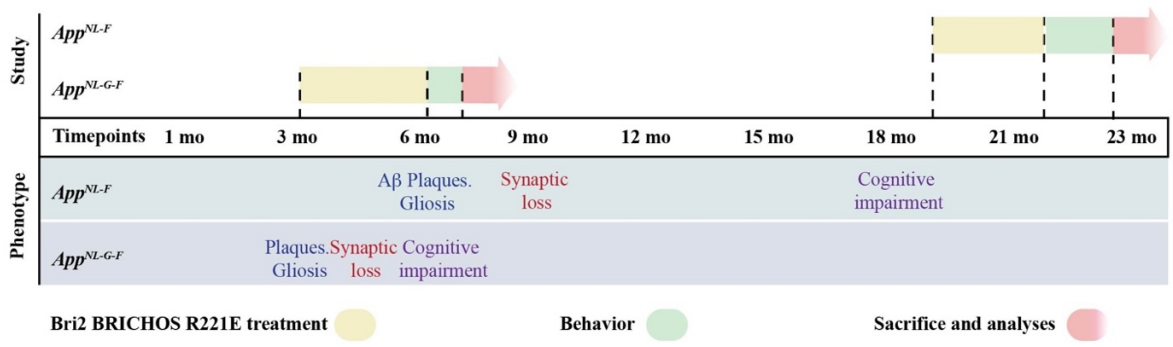

Figure 11. Scheme of the rh Bri2 BRICHOS R221E treatment study design in *App*$^{NL-F}$ and *App*$^{NL-G-F}$ mouse models. Study in Paper IV included both *App*$^{NL-F}$ and *App*$^{NL-G-F}$ mouse models treated by intravenous injections with rh Bri2 BRICHOS R221E for 10 weeks at 19 months and for 12 weeks at 3 months age, respectively. Several behavioral experiments were performed immediately after the treatments before the mice were sacrificed in order to collect samples that were analyzed by biochemical methods. The upper panel shows the timeline of the study regarding treatment, behavior and biochemical and histological analyses in different colors. The phenotypes associated to each mouse model are described in the sections below the timepoints.

In **paper IV** we treated a total of 10 female *App*$^{NL-F}$ mice for 10 weeks from the age of 19 months while the studies in 12 female *App*$^{NL-G-F}$ mice were conducted starting at the age of 3 months. After the completion of the treatments, a set of behavioral experiments described in section 4.4. were conducted before subjecting the brains to different biochemical analyses.

4.4 BEHAVIORAL PARADIGMS

In **Paper IV** various behavioral tests were performed, that target a variety of types of learning, fear, mobility and memory functions, to assess the behavioral effects associated with the treatment.

4.4.1 Y-maze

The Y-maze test is used for evaluation of the short-term working memory, which involves several parts of the brain, including the hippocampus, septum, prefrontal cortex and basal forebrain (309, 310). The Y-maze test is based on the natural curiosity of the mice to explore novel environments. The apparatus is made of gray plastic consisting of three identical opaque compartments, with high walls, that extend from a center platform forming a Y-shape. After the mice are introduced to one of the arms as starting point, they are allowed to explore the arms freely for 5 minutes. Typically, they would tend to explore a new arm of the maze instead of returning to the one that they previously visited (Figure 12). The number of entries is considered when the mouse hind limbs are within the arm and are tracked with a video camera to analyze the percentage of alternation of entries into different arms. A mouse that suffers memory deficits would show reduced spontaneous alteration between the arms.

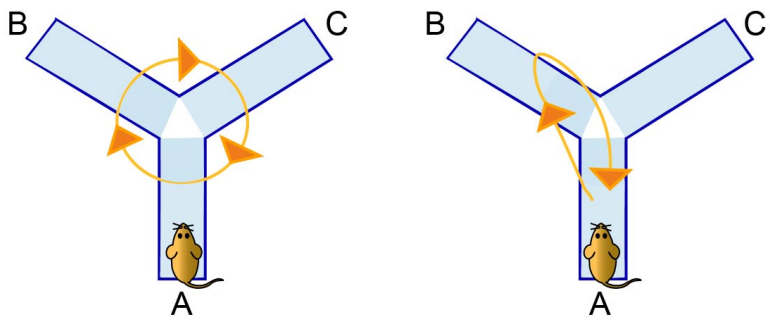

Figure 12. Illustration of Y-maze test. Example of correct (left image) and incorrect (right image) alternations of entries indicated by arrows.

4.4.2 Elevated plus maze

AD patients are reported to have anxiety-like behavior; therefore, we have used elevated plus maze (EPM) test to assess anxiety-like behavior. The paradigm consists of a "+" shape platform with two oppositely positioned closed arms and two oppositely positioned open arms, and a center area (Figure 13). After the mice are placed in the center of the maze, they are allowed to freely explore the arena and their movements are recorded with a video camera. What we evaluate in this test is their innate preference for being in the closed arms over their fear to be in the open arms, which gives an estimation of their anxiety state.

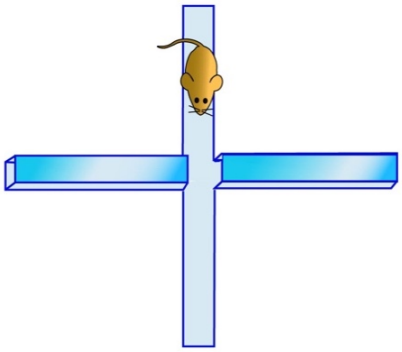

Figure 13. Schematic representation of elevated plus maze platform. The instrument consists of two open and closed arms 40-50 cm above the ground. The mouse is placed in the middle of the platform and allowed to explore freely the environment.

4.4.3 Open field and novel object recognition

The purpose of the open field (OF) test is to measure the exploratory behavior and the general activity of rodents and anxiety assessment, and the outcome is activity or "movement" in measures such as distance moved, change of activity during time, speed or freezing and time spent moving. The OF is commonly a square or rectangular box, that allows the mice to spend time in the corners, with high walls that prevent the animals to escape. In **Paper IV**, the OF test precedes the novel object recognition (NOR) test (Figure 14) used to analyze the discrimination ability of the mice for familiar and novel objects added in the chamber, and both can be performed in the same arena. The first day (OF test), the mice are individually

introduced into the box or arena where they are allowed to explore the area for 5 to 10 minutes (311), the so called habituation phase.

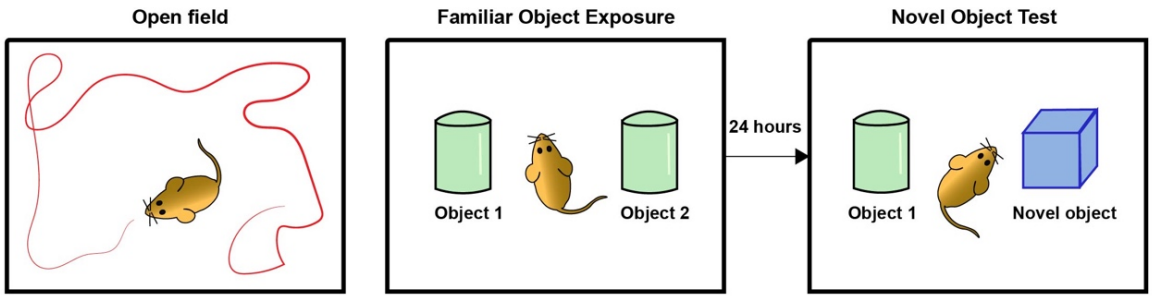

Figure 14. Open field (OF) and novel object recognition (NOR) tests. On the left, the mouse explores the arena during the habituation phase and their activity is recorded. In the NOR test, after a break period, two identical objects (familiar) are added in the arena and a single animal is placed in the OF for a few minutes. 24 hours after, the animal is returned to the arena where there are now two objects, one identical to the familiar one and the other is novel.

In the familiarization phase, two familiar objects are placed opposite to each other and equidistant from the walls of the box. After a gap of 24 hours in the test phase, one of the objects is replaced with a novel object in the same position as the previous one, and the mice are allowed to explore the arena in the presence of the familiar object and the novel object. Normally, the animal with intact recognition memory would spend more time exploring the novel object. The animals are recorded with a video camera for analyzing different parameters associated with these tests.

4.4.4 Morris water maze

The Morris water maze (MWM) was performed in **paper IV** to evaluate the hippocampal-dependent spatial learning and memory of mice (312, 313). It is done in a circular tank that contains water made opaque by adding milk or other substance that helps to hide the platform and track the mice. This tank is divided into four quadrants and on the walls of the room there are usually some stationary geometric cues that are visible for the mice while they swim (Figure 15). During the performance, it is important to control the light of the room, that should be soft and diffuse, as well as the temperature of the water (about 25°C). The task the mice need to follow is simple. During the training trial, the mice are placed in one of the quadrants of the pool, as starting point, and they are allowed to swim to find the platform (located in the target quadrant), whose location is identified using spatial memory. The duration of the training trial can vary.

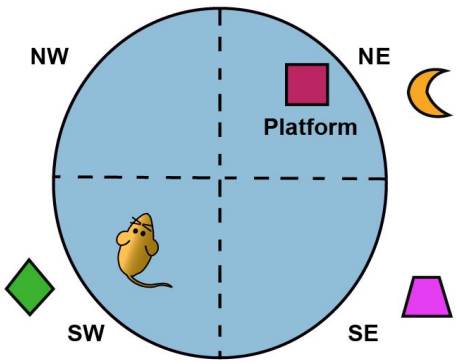

Figure 15. Morris water maze (MWM) test illustration. The MWM consists of a pool filled with water and virtually divided into four identical quadrants identified with Northeast (NE), Northwest (NW), Southeast (SE) and Southwest (SW). A platform is placed inside the tank (Northeast quadrant in the image) and some visual cues are added in the room.

In the study in **paper IV**, four trials per day during four consecutive days were completed. The learning of the mice during the training sessions is measured by the latency time that they spend to find the hidden platform. After the training sessions, the mice are subjected to probe test without the platform present in the target quadrant, and they are evaluated for their retention of spatial memory by tracking the time spent in the target quadrant. The mice that spend more time in the target quadrant show better retention memory.

4.4.5 Fear conditioning

This test is used to assess the amygdala and hippocampus-dependent fear memory in rodents. In **paper IV** we performed fear conditioning (FC) test to investigate the aversive learning and memory response in App^{NL-F} mice. The freezing behavior, defined as complete immobility, is used to study the fear and learning memory after the mice are exposed to an auditory cue followed by an electric footshock.

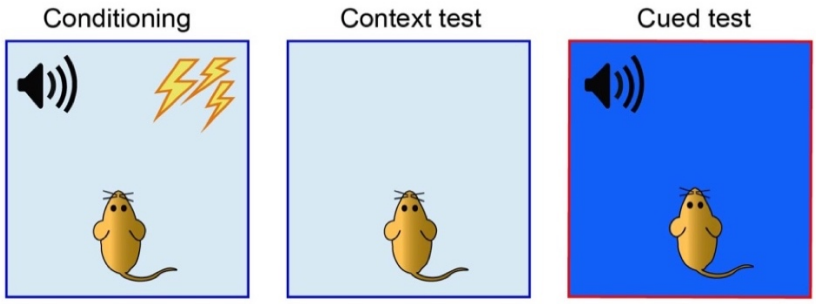

Figure 16. Scheme for the fear conditioning (FC) test. On the conditioning day (left), mice are subjected to two stimuli (tone and electric shock); the following day (context test) the mice returned to the same chamber and they are let to explore the maze. One day later, the cued test is performed by placing the mice in a different chamber where they received a tone stimulus.

In the most common used procedures, during the first day (conditioning day), the mice are placed inside a chamber and they receive a conditioned stimulus (tone) followed by an aversive unconditioned stimulus (mild foot shock). One day after (context test day), the mice are exposed to the same chamber where they explore the context without receiving any footshock. During the cued test day, after an interval of usually 24 hours, the animals are placed inside a

novel test chamber of distinct appearance (wall color, floor texture and odor), where they explore the box and immediately after, they receive a tone stimulus (Figure 16) (313). The mice are recorded by a video camera to trace their freezing behavior.

4.5 FOCUSED ULTRASOUND TECHNIQUE FOR OPENING THE BLOOD BRAIN BARRIER

The BBB opening to allow drug delivery to the brain can be achieved with the application of FUS in combination with MBs, as explained in section 2.6. This technique applied to enhance the BBB permeability stands out from others techniques as the most promising approach due to its non-invasive, safe, transient and localized characteristics (314).

There are several parameters to take into account in order to make BBB opening a safe method: the frequency of the pulse, the acoustic pressure, the duration of the pulse, the frequency of the pulse repetition and the total sonication time. All these parameters have been optimized in small animals like mice and rats.

The ultrasound instrument adapted to mice that we used in **paper II** consists of a single-element FUS transducer that reproduces the ultrasound waves. Below and inserted in a central hole, there is a pulse-echo transducer that is used to detect the cavitation signal without sending any pulse during the sonication, so it does not cause interference to the focused ultrasound transducer. Under the ultrasound transducers, a container with cone shape is filled with degassed and distilled water. The signals received by the pulse-echo transducer are amplified by the pulse-receiver, which is connected to a digitizer in order to acquire the acoustic emissions from the MBs, that are recorded in real-time (Figure 17) (285). Before the sonication treatment, the anesthetized mouse is placed on a heating pad that keeps a constant temperature and a stereotactic apparatus used to immobilize the head of the animal. To minimize the mismatch, the hair present on the head of the mice is removed with a depilatory cream. The administration of the MBs and the compounds is done through the tail vein of the mice. The fact that there is no need for surgery, the MBs are injected intravenously, makes this technique suitable for repeated treatments.

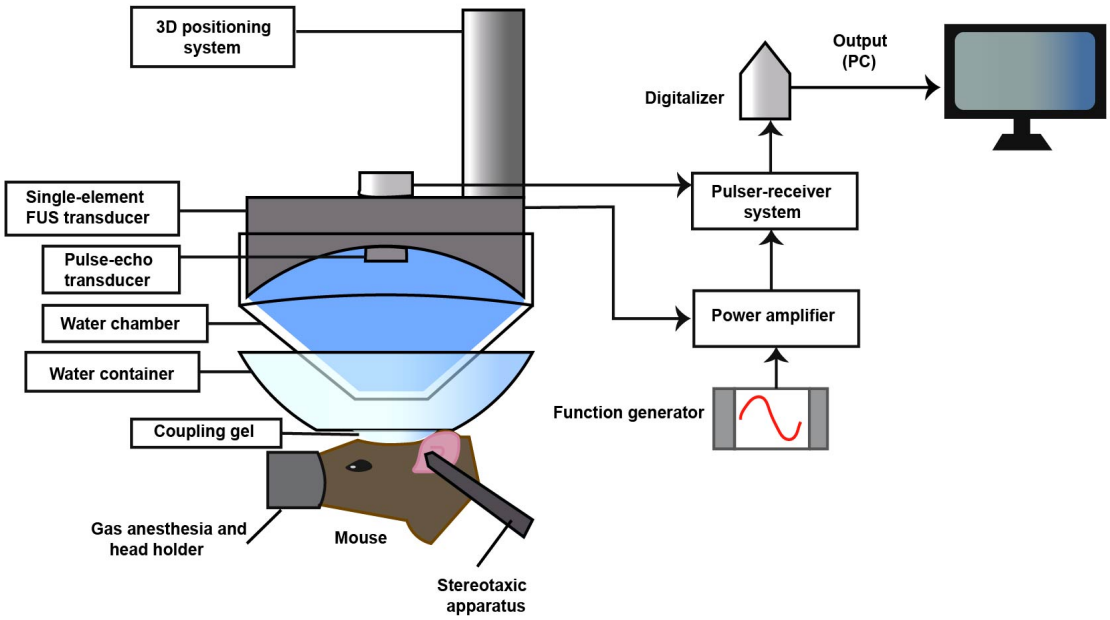

Figure 17. Illustration of the general FUS set up for sonication of the cerebral BBB on mice. Figure adapted from Choi J.J. et al., 2011 (315).

During the sonication, the FUS energy focuses on a targeting region, which is affected by the ultrasound beam while the tissue in the surrounding areas remains unaffected. In the region where the opening occurs, the acoustic pressure is maximum and if the ultrasound is calibrated, the cavitation only happens in the targeting point. Magnetic resonance imaging (MRI) allows to control and visualize the spot of the BBB opening in the brain. This is accomplished by using an intraperitoneal administered MRI-contrast agent that does not cross the BBB under normal conditions. The BBB disruption visualization by MRI is carried out approximately 20 to 30 minutes after sonication. In our studies, we took T_1 -weighted images and we could see that the contrast of the MRI images is enhanced only at the position where the opening occurs (292). In addition, it is possible to obtain T_2 -weighted images with the purpose of studying the safety of the sonication, providing us information about the presence or absence of microhemorrhages or edema at the sonicated region.

Opening the BBB requires energy to overcome the attenuation and diffraction perceived from the skull of the animal. Hence, FUS is used in conjunction with MBs. The cavitation of the MBs is the mechanism by which FUS induces the localized and efficient BBB opening at the cerebral vasculature. There are two types of cavitation depending on the acoustic pressure: stable and inertial (also called transient) cavitation (285). The stable cavitation is associated with low pressures, while when high pressures are applied and the amplitude increases, the MBs enter in a more violent bubble oscillation that is characterized by the inertial cavitation and this can lead to bubble collapse. Stable cavitation is required for the BBB opening while inertial cavitation must be avoided due to its association with microdamage (290). During stable cavitation, the MBs induce mechanical BBB disruption by exerting shear forces on the endothelium. This stress depends on the amplitude and the frequency of the ultrasound, as well

as on the size of the microbubbles compared to the blood vessels (299). The inertial cavitation is characterized by bubble instability and is an indicator of cellular damage due to the higher pressures to induce this phenomenon (316). The opening of the BBB is not permanent; it lasts for a few hours, and when the BBB closes it does not loss its functionality (317). Applying this method, we can transport virtually any drug inside the brain. The different transport mechanisms induced by FUS treatment were described in section 2.5.

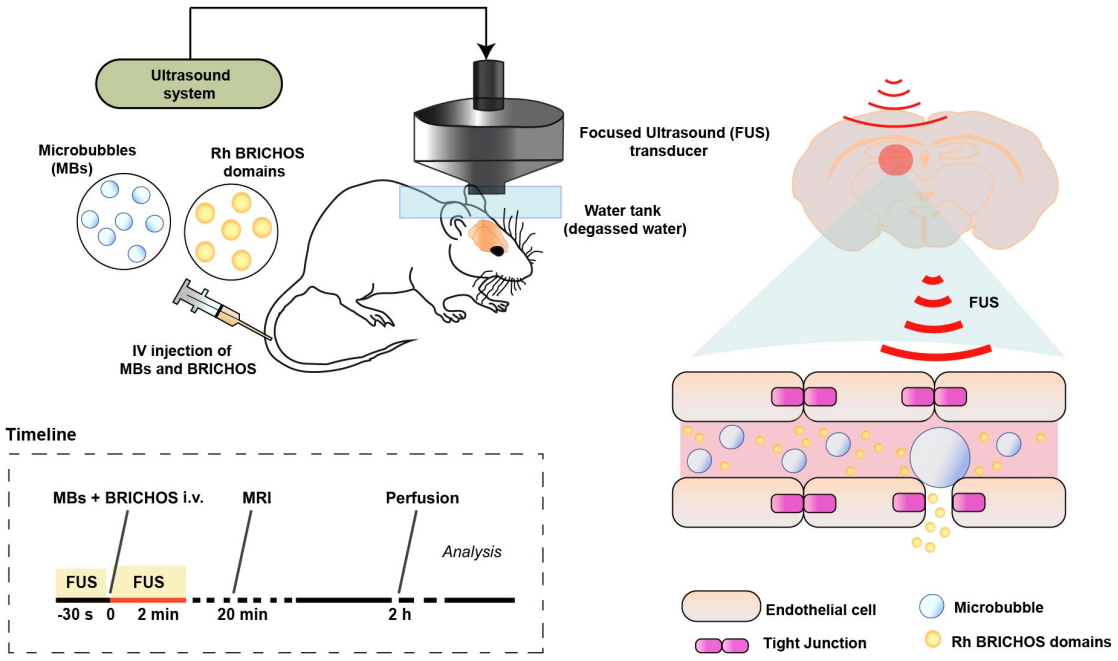

Figure 18. Overview of the FUS in combination with MBs and administered BRICHOS domains used in paper II. On the upper left panel, rh BRICHOS domains and MBs were administered in the tail vein of the mouse immediately before FUS sonication. The right panel shows how the BBB disruption between endothelial cells due to the cavitation of the MBs allows the rh BRICHOS domains to enter the brain. The different experimental steps followed in **paper II** are summarized in the Timeline box at the lower left.

In **paper II**, we treated wt mice with FUS in combination with MBs to allow rh BRICHOS domains to be delivered over the BBB. Figure 18 illustrates a summary of the experimental workflow followed in the study. This approach aimed to sonicate the left hippocampus of each mouse and leaving the right hemisphere (non-sonicated) as a control, with the advantage of reducing the variability among animals. Prior the administration of MBs and rh BRICHOS domains, sonication is applied to get the baseline of acoustic response needed to quantify the cavitation dose. After that, the intravenous injection of MBs and the proteins were followed by a sonication of 2 minutes. During that time, the intravenously administered MBs oscillate within the blood vessels. Approximately 2 hours later, the mice were sacrificed, and the brains collected to evaluate the delivery of rh BRICHOS domains in the sonicated region and the non-sonicated control hemisphere.

4.5.1 The microbubbles (MBs)

The MBs applied in FUS treatments are small bubbles of more than one micrometer diameter but smaller than one millimeter diameter. Due to their small size, they are capable to travel through the smallest capillaries in the body. They all have a common composition which is a core made of inert gas encapsulated by a biocompatible and stable shell. The MBs used for the studies in **papers II** and **III** are composed by a shell made of lipids. The lipid shell of MBs has been inspired by nature and mimics lipid vesicles. The shell of MBs can alternatively be made of proteins (albumin, lysozyme, avidin), surfactants (TWEEN, SPAN) or polymers, or a combination of these materials (318). The shell protects the innocuous gas in the core, which comprises most of the particle volume (Figure 19). MBs are unstable in an aqueous media, and the gas helps to stabilize the shell.

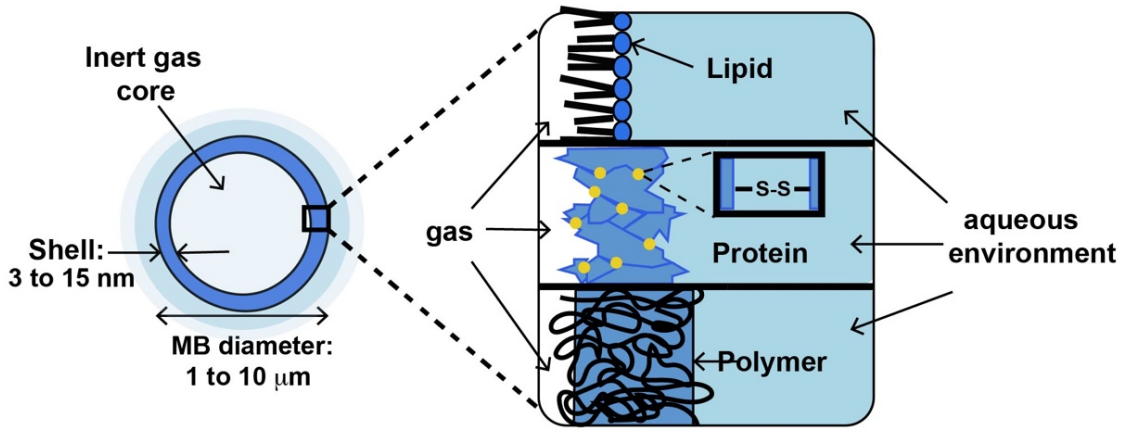

Figure 19. Cartoon showing the microbubbles general composition and structure. MBs diameter is between 1 to 10 μm and their shell that is composed by different materials: lipids, proteins or polymers, is 3 to 15 nm thick. The shell, surrounded by aqueous media, encapsulates the gas core which stabilizes the MB. Adapted from © 2019, Sonaye H.V., Shaikh R.Y., Doifode C.A. Published in Sonaye H.V. et al., Intechopen, 2018 under CC by 3.0 license. Available from: http://dx.doi.org/10.5772/intechopen.87157.

In the case of lipid MBs, their saturated phospholipids reach a low surface tension that maintains a gel-like state, and in addition, the monolayer is kept together by weak hydrophobic and van der Waals interactions between the acyl chains (318).

The size of the MBs has an important impact in the opening of the BBB. MBs diameter is inversely proportional to the resonance frequency when all parameters remain the same. Regardless of the shell property, the resonance frequency thus increases when the size of the bubble decreases. The threshold for the BBB opening by inertial cavitation is at 0.45 MPa for bubbles of 1-2 μm diameter and for bubbles with diameter size of 4-8 μm 0.30 MPa is required (290). The diameter of the capillaries in the murine brain is between 4 to 8 μm. In our study in **paper II**, the bubble size is 4-5 μm in diameter, and the FUS frequency used was 1.5 MHz. Not only the size but also the dose of MB´s seems to affect the BBB opening as larger doses increase the opening (291). In general, the opening of the BBB is affected by many parameters, so achieving optimal enhancement of BBB permeability requires fine-tuning of both ultrasound set-up and ultrasound energy.

In-house made lipid microbubbles

The monodisperse lipid MBs used in **paper II** were produced in the laboratory on the same day or one day before the experiments. The lipid monolayer is spontaneously formed by dissolving in phosphate buffer a mixture of 90 mol% of 1,2-distearyol-sn-glycero-3-phosphocoline (DSPC), and 10 mol% of 1,2-distearyol-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethyleneglycol)2000] (DSPE-PEFG2000) that prolongs the half-life of the MBs circulating in plasma. The gas contained in the core of the MBs is perfluorobutane. When these MBs are activated, the bubbles 4-5 μm of diameter can be isolated from a polydisperse distribution (285).

Commercial lipid microbubbles

MBs have been clinically used as ultrasound imaging contrast agents in the clinic for more than two decades and they are also used as carriers for drug delivery for therapeutic applications. There are several lipid MBs that have been approved by the US Food and Drug Administration (FDA): Definity® (Lantheus Medical Imaging), Imagent® (Alliance Pharmaceutical), Sonazoid™ (Amersham Health) and Sonovue® (Bracco Diagnostics). Most studies applying FUS treatment have used commercial MBs, which are normally polydisperse, for inducing BBB opening. In the current thesis we have treated the animals with both Definity® and Sonovue®.

In **paper II** some mice were administered with Definity®, which are polydisperse MBs with the following size distribution; mean diameter is 1-3 μm, maximum 20 μm and 98% of the bubbles had a diameter under 10 μm. The gas core is octofluoropropane and it is encapsulated by lipid microspheres. They are encapsulated in a vial containing 1.2 x 10^{10} bubbles/ml. During studies in **paper III,** we administered Sonovue® together with rh Bri2 BRICHOS domains without FUS application. These MBs are composed of the innocuous gas sulphur hexafluiride protected by a shell of lipids: macrogol 4000, DSPC, 1,2-dipalmitoyl-sn-glycero-3-phosphorylglycerol sodium (DPPG) and palmitic acid. In the case of Sonovue®, their mean diameter is about 2.5 μm where about 90% of them have a diameter less than 6 μm and 99% less than 11 μm, and their reported concentration is 1-5 x 10^{8} microbubbles/ml (319). After a single intravenous injection of Sonovue®, the calculated mean half-life is 12 minutes (range 2 to 33 minutes) being the peak concentration in blood 1 to 2 minutes after administration (320). Other studies have reported a half-live of 5 minutes for Definity in blood circulation (321, 322).

Both Definity® and Sonovue® have been shown to have similar effects in ultrasound and BBB-opening applications (323, 324).

4.6 IMMUNOHISTOCHEMICAL ANALYSES

One of the most used techniques in the current thesis, especially as part of studies in **papers I-III**, is immunohistochemistry (IHC). The immunohistochemical analyses have been key to shape our understanding of our different approaches of rh BRICHOS domains delivered into

the brain, together with western blotting, immunoprecipitation and ELISA. For detection of rh proSP-C BRICHOS domains we did immunostaining using anti-proSP-C antibody, which recognizes the BRICHOS domain contained in the proSP-C protein; while for detecting the rh delivered Bri2 BRICHOS domains we used an antibody against a six amino acids residue-tag, an AU1 tag (DTYRYI), that was genetically added to the C-terminal region of rh Bri2 BRICHOS and recombinantly produced, in order to enable discrimination of the recombinantly administered Bri2 BRICHOS domain from the endogenous mouse Bri2 protein present in brain.

In **paper I**, the mouse brains were divided into the two hemispheres, one hemisphere was used for western blot and ELISA detection while the other hemisphere was kept for IHC analyses. Alternatively, in **paper II**, after collection of the mouse brains, the whole organ was dehydrated and embedded in paraffin to further investigate by IHC the delivery of rh BRICHOS domains after FUS treatment. This same approach was followed in **paper III** to study the delivery of Bri2 BRICHOS domains in the presence of MBs. The sections embedded in paraffin were first deparaffinized, rehydrated and the antigen retrieval was applied. Thereafter, the sections were blocked to avoid non-specific binding of the antibodies and incubated with primary antibodies. Thereafter and following a washing step, secondary antibodies were added to the sections. In **paper I**, DAB staining was used for stain development, while in **papers II** and **III**, a red chromogen substrate of alkaline phosphatase was added which results in a magenta-red color possible to visualize both by light and fluorescence microscopy. In all cases, control samples were used in order to check for the possibility of unspecific staining or background. Finally, the images were acquired by a Nikon Eclipse E800 light microscope using NIS Elements software.

5 RESULTS AND DISCUSSION

"Wisdom begins in wonder." -Socrates

Despite the progress in AD research that has been made in the last years, a long row of clinical trials has failed and up to date, there is no effective treatment for this disease. In the search for new therapeutics, it has been identified, as described in section 2.3.3., that the molecular chaperone domain BRICHOS has a unique function: it binds to the surface of fibrils that generate the amyloid plaques in AD, being a potent inhibitor of Aβ aggregation (12, 13, 16), and, importantly, it has been shown that rh BRICHOS domains can reduce neurotoxic effects on mouse hippocampal slices (14, 18, 19, 233) and in fruit flies brains (17, 18). Here, we sought to obtain answers on how we can use rh BRICHOS to develop a drug for Alzheimer´s disease, testing BRICHOS in mouse models. Thus, our approach was to study if BRICHOS, administered into the circulation, could enter the brain and inhibit the Aβ toxicity. The general findings of the work presented in this thesis can be summarized from three aspects.

5.1 A FIRST STEP TO A TREATMENT STRATEGY: STUDIES ON BBB PASSAGE OF RH BRICHOS DOMAINS IN WILD TYPE MICE (PAPER I).

One major obstacle for a drug to treat neurodegenerative diseases is its ability to reach the brain parenchyma, since the BBB efficiently hinders the passage of many compounds into the CNS. Many of the drugs used in AD treatment projects involve molecules that are too large to pass the BBB (269). We started by asking if rh BRICHOS constructs could pass over the BBB and reach the brain parenchyma, after peripheral administration. To assess that, in **paper I** we expressed and produced rh BRICHOS domains from proSP-C and Bri2 proteins. ProSP-C is only produced in the alveolar type II epithelial cells (325) and not in the brain like is the case for Bri2 (216), and we sought to evaluate possible differences in permeabilities between the two BRICHOS domains.

Rh Bri2 BRICHOS domains was produced and purified using the NT* solubility tag. As Bri2 is an endogenous protein present in the brain, we modified rh Bri2 BRICHOS by adding a 6 amino acid residue AU1 tag (DTYRYI) to the C-terminal end of BRICHOS domain to enable the differentiation between the administered rh Bri2 BRICHOS and endogenous mouse Bri2. By SEC, we could isolate distinct quaternary structures of rh Bri2 BRICHOS domain, in order to not only test the BBB passage of crude mixture of monomers, dimers and larger oligomers, but also of each isolated specie, which have been found to play different roles against Aβ42 fibril formation and neurotoxicity (233). Before administration of the purified rh Bri2 BRICHOS-AU1 into the mice, we confirmed by Thioflavin-T assay that it had similar inhibitory effects on Aβ42 as the unmodified wt rh Bri2 BRICHOS.

To determine the efficiency of the different BRICHOS domains to pass the BBB as well as if any acute toxicity could be observed, we chose male wt mice age 8 to 10 weeks, that were injected intravenously with rh Bri2 BRICHOS-AU1 and proSP-C-BRICHOS proteins at doses ranging from 10 to 50 mg/kg and blood, brain and CSF samples were collected at different time points. Control animals for these experiments were administered with PBS. Pharmacokinetics analysis revealed that the BRICHOS domain from proSP-C has a somewhat longer serum half-life compared to rh Bri2 BRICHOS-AU1 mixture of oligomers, dimers and monomers, and its individual assembly state species. Despite the similar half-lives of the different assembly states of Bri2 BRICHOS, their intra-brain distribution may vary.

In order to investigate to what extent rh BRICHOS can be found in the brain, we used immunohistochemistry, ELISA and western blots. First, we evaluated the blood-cerebrospinal fluid barrier (BCSFB) permeability of BRICHOS and both proteins were detectable, even though the western blot bands for rh proSP-C BRICHOS were weaker. Although we could detect proSP-C BRICHOS in the CSF, it was not found in the brain parenchyma by western blot or immunohistochemistry. ProSP-C BRICHOS forms mainly homotrimers in solution (201, 234), a phenomenon that may influence the passage across the BBB. Unlike rh proSP-C BRICHOS, rh Bri2 BRICHOS-AU1 mixture was detectable in the brain parenchyma by immunohistochemical analyses. For rh Bri2 BRICHOS-AU1 detection by western blot, we used two antibodies; one antibody binds to the Bri2 BRICHOS domain and the second antibody recognizes the AU1 tag. Both of them detected Bri2 BRICHOS bands corresponding to the size of 17 kDa. Not all the analyses of the animals administered with the crude mixture of rh Bri2 BRICHOS domain were positive. At a dose of 20 mg/kg, we found that the uptake was more effective after 2 hours than when administered a higher dose (50 mg/kg) of rh Bri2 BRICHOS-AU1. In a similar manner, we could not detect higher amounts of the protein in the brain when the duration between the administration and the analyses were longer than 2 hours. Considering that, it is not surprising that when we administered the larger oligomeric species of rh Bri2 BRICHOS domain, which consists of about 20 subunits, we could not detect them in the brain, but we observed uptake by both western blot and immunohistochemical analyses after administration of the monomer and dimers. As the different assemblies of rh Bri2 BRICHOS have different mechanisms of action, it is conceivable that they also behave in a different manner when it comes to their ability to cross the BBB. In addition to that, a higher concentration of administered crude Bri2 BRICHOS-AU1 (50 mg/kg) could shift the equilibrium towards oligomers, as has been previously observed in mouse serum (233), and supported by SDS-PAGE under non reducing conditions. The dimers and monomers apparently are the most efficient species to cross the BBB.

Intriguingly, we observed the strongest immunostaining for rh Bri2 BRICHOS inside the ependymal cells in the choroid plexus epithelium and in cells in the cerebral cortex, but the specific intracellular localization was not studied. There are regions of the brain with different permeabilities, the endothelium of the blood-CSF barrier located in the ventricles and the choroid plexus is fenestrated and therefore are more permeable (326). This could explain the presence of proSP-C BRICHOS in the CSF.

We were able to quantify the amounts of rh Bri2 BRICHOS-AU1 in the brain by sandwich ELISA. The concentrations detected in the brain parenchyma after a dose of 20 mg/kg varied between 120 and 880 nM, that is higher than the soluble Aβ40 and Aβ42 found in human AD brains (30-120 pM) (327). Sub-stoichiometric concentrations of rh Bri2 BRICHOS domains can prevent Aβ42 induced neurotoxicity in hippocampal preparations (233), in particular Bri2 BRICHOS monomers, which apparently are the most BBB permeable species. According to our ELISA results, 0.1% to 1% of the total amounts of injected rh Bri2 BRICHOS-AU1 reaches the brain. Considering that antibodies result in a maximum brain concentration of 0.1% of the total dose (272, 328, 329), the data on Bri2 BRICHOS permeability are promising. The passage of antibodies or peptides occurs by transendothelial diffusion (270), transcytosis or endocytosis (330), while the mechanisms by which Bri2 BRICHOS is transferred to the brain remains to be investigated. Non-lipophilic compounds can be transported into the brain by receptor-mediated transcytosis and probably there are receptors that have not been identified yet. Bri2 BRICHOS is expressed in peripheral tissues and in the CNS (331). Considering this, it is possible that Bri2 BRICHOS could bind an unidentified receptor and be transported into the brain. Single cell RNA-sequencing revealed that Bri2 gene (*ITM2B*) expression is specially high in the pyramidal neurons in the CA1 region of the hippocampus in mouse (332) and in the same cells in the CA3 and CA4 hippocampal regions in humans (215, 216, 331). These cells are essentially the same as the ones that take up rh Bri2 BRICHOS (**paper II**), suggesting that Bri2 localized to the plasma membrane may act as a receptor for circulating Bri2 BRICHOS. This possibility deserves attention in future studies.

It is of importance to consider that the presence of rh Bri2 BRICHOS-AU1 in the choroid plexus and CSF contribute to the detection of the protein in the brain homogenates, although Bri2 BRICHOS was also found in other brain regions by immunohistochemistry. Nevertheless, our findings show that rh Bri2 BRICHOS-AU1 can reach the brain parenchyma after peripheral administration.

5.2 STRATEGIES TO ENHANCE BBB PERMEABILITY USING THE BRI2 BRICHOS DOMAIN (PAPERS II AND III).

As we found in **paper I** that rh Bri2 BRICHOS but not proSP-C BRICHOS could pass to the brain, next we sought to increase the passage over the BBB of both rh BRICHOS domains. FUS technology in conjunction with intravenous injection of lipid MBs was particularly attractive since it has been successfully applied to deliver macromolecules (dextrans) of up to 70 kDa (333), and has been described as a promising technique able to deliver drugs over the BBB, inducing its opening locally, transiently and non-invasively (285, 334-336). It has been recently reported that ultrasound together with MBs increased the levels of Aducanumab antibody in AD mouse models and that this combination improved the cognitive functions compared with the only administration of the antibody (337). Thus, in **paper II** our goal was to apply FUS technology in combination with MBs to disrupt the BBB and enable the passage of rh Bri2 BRICHOS-AU1 and rh proSP-C BRICHOS proteins into the brain of wt mice.

During the first part of the study, we administered MBs generated and isolated in-house, and for the second part of the study when we analyzed the delivery by western blot analyses and performed quantifications of the protein in the brain, we used the FDA-approved microbubbles Definity®. The two types of MBs used have equivalent effects in the opening of the BBB (324). The hippocampus is severely affected in AD (338) and we therefore targeted this region by FUS. We used adult female wt mice and, initiated the study with an intravenous rh proSP-C BRICHOS dose of 1 mg/kg, since we expected to have a higher delivery compared to that obtained in **paper I**. However, we failed to observe any delivery and therefore, we increased the dose to 10 mg/kg, which was sufficient to achieve delivery rh proSP-C BRICHOS to the targeted brain region, although it was not able to cross the BBB when administered without the application of FUS and MBs (**paper I**). Rh Bri2 BRICHOS-AU1 successfully crossed the BBB at the ipsilateral side, and surprisingly, we found that the protein was present in the contralateral side, showing a practically identical pattern in both hemispheres. We monitored the opening procedure with MRI which confirmed that the opening occurred only in the targeted left hemisphere, although we detected rh Bri2 BRICHOS-AU1 in both brain hemispheres. To prove that the staining was not due to unspecific antibody binding, we stained for the AU1 tag in the brain of mice that were FUS sonicated and treated with rh proSP-C BRICHOS and MBs, which gave no signal.

Staining with an antibody that recognizes mature neurons showed that both rh proSP-C BRICHOS and rh Bri2 BRICHOS-AU1 were internalized by neuronal cells. In the case of rh proSP-C BRICHOS, we could visualize intracellular staining in cells located in the granular cell layer of the dentate gyrus in the sonicated hippocampus, while the internalization of rh Bri2 BRICHOS-AU1 occurred predominantly in the pyramidal neurons in the CA1 and CA3 regions, but also in the cortex. We next aimed to identify the intracellular localization of rh BRICHOS domains. However, the colocalization studies of rh BRICHOS domains with early endosomes did not provide us with clear answers. Several cellular uptake mechanisms have been described (339) and the poor endosomal colocalization observed makes it unlikely that BRICHOS domains are carried via the endosomal pathway. The mechanisms by which the internalization occur are still unknown and should be an interesting topic for future studies.

These data match with those obtained in **paper I** from two different aspects: they further support the intracellular staining of rh Bri2 BRICHOS-AU1 seen in the cortex and striatum, and they confirm that rh proSP-C BRICHOS and rh Bri2 BRICHOS differ qualitatively when it comes to BBB permeability. One way to rationalize the unexpected distribution of Bri2 BRICHOS domain in both the FUS targeted and non-targeted hemispheres is that the MBs might have an effect on the BBB permeability of rh Bri2 BRICHOS domain, facilitating its passage. The transport mechanisms by which rh Bri2 BRICHOS domain crosses the BBB has not been studied in the present thesis, however, as mentioned in the previous section, the existence of a transporter that facilitates Bri2 BRICHOS transport from the periphery to the CNS is conceivable, and it needs experimental testing.

Paper III explored whether the MBs used in the FUS experiments contributed to the phenomenon observed in **paper II**, where FUS in combination with MBs led to an increased BBB permeability of rh Bri2 BRICHOS-AU1 in both brain hemispheres. MBs are considered theranostic agents (ie. have combined therapeutic and diagnostic properties); as drug delivery systems in combination with ultrasound and acting as imaging probes. Lipid nanoparticles have been used associated to drugs or encapsulating them, increasing their distribution and bioavailability (340).

To probe their effects on BBB permeability, we used commercial MBs and administered them intravenously into wt mice followed by rh Bri2 BRICHOS-AU1 at different doses ranging from 1, 10 and 20 mg/kg, and then compared the delivery of rh BRICHOS in those mice with control mice. In agreement with previous findings, rh Bri2 BRICHOS-AU1 was detected in mouse brains collected 2 hours after the double injections (MBs and rh Bri2 BRICHOS) (Figure 20A, B). Although no specific cellular marker was used, the abundant positive staining followed a similar pattern in the cortex, hippocampus and choroid plexus as the observed in **paper II**. Control experiments were included resulting in no signal detected for the AU1 tag. In addition, we assessed the effects of MBs on BBB permeability by fusing the globular protein mCherry to rh Bri2 BRICHOS (341) to facilitate the detection of the administered rh BRICHOS domain (341). The analyses revealed a red fluorescent signal in the striatum region of two mice administered with MBs and rh Bri2 BRICHOS-mCherry, which was not found in any mouse injected only with rh Bri2 BRICHOS-mCherry (Figure 20C, D). This supports our hypothesis that peripherally administered Bri2 BRICHOS crosses the BBB when combined with MBs, even when it has been fused to a globular protein of 30 kDa. The mechanism(s) by which rh Bri2 BRICHOS-AU1 or rh Bri2 BRICHOS-mCherry in the circulation cross the BBB remains to be clarified, but we hypothesize that MBs might affect the half-life of rh Bri2 BRICHOS, even when linked to other proteins. Other factors may contribute as well, eg MBs may prolong the bioavailability and decrease clearance *via* decreased heart rate after administration (342).

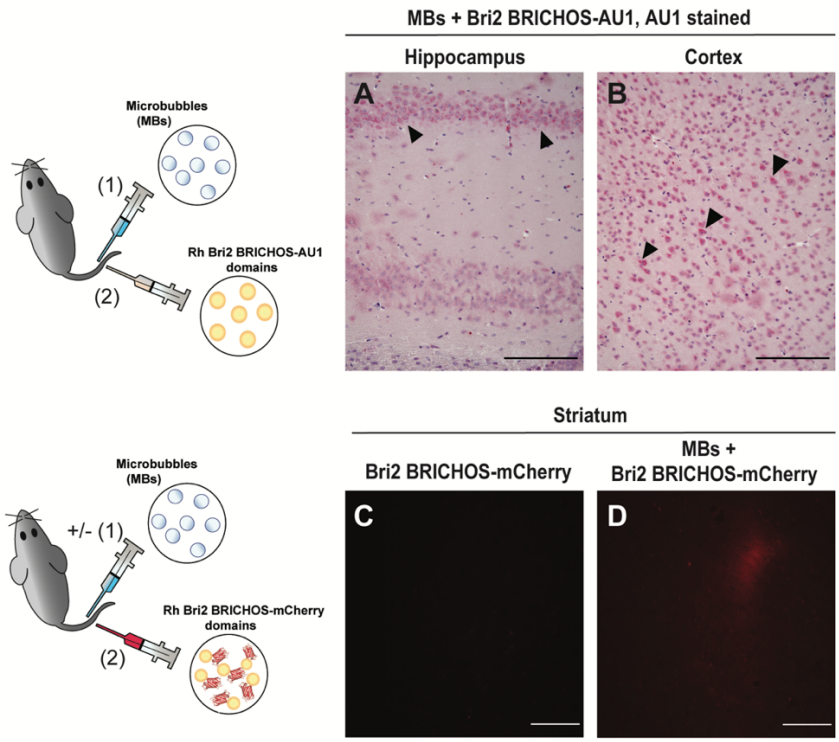

Figure 20. Visualization of rh Bri2 BRICHOS-AU1 or rh Bri2 BRICHOS-mCherry uptake in the brain parenchyma after systemic MBs administration. (A, B) Immunohistochemical visualization of rh Bri2 BRICHOS-AU1 in the hippocampus (A) and cortex (B) of a mouse treated with rh Bri2 BRICHOS-AU1 and MBs. (C, D) Fluorescence imaged in the striatum region after intravenous injection of rh Bri2 BRICHOS-mCherry with (D) or without (C) MBs. Sizes of scale bare are 50 μm for panels A, B; and 100 μm for panels C, D.

Our next step was to evaluate the uptake of rh Bri2 BRICHOS-AU1 in both brain hemispheres by western blot and, in a more quantitative manner, by sandwich ELISA. We analyzed brain samples of mice that were administered 10 mg/kg of rh Bri2 BRICHOS with the application of FUS and MBs (**paper II**) and when only MBs are used (**paper III**). As judged by the western blot analyses in both studies, bands migrating as rh Bri2 BRICHOS-AU1, that were not present in control samples, had similar intensities in the two hemispheres, supporting our observations regarding equivalent amounts of Bri2 BRICHOS-AU1 from the immunohistochemical analyses. According to ELISA, rh Bri2 BRICHOS-AU1 concentrations were identical in the contralateral and ipsilateral sides and about 0.4% of the total amount administered passed into the brain parenchyma when FUS in conjunction with MBs was applied. Notably, the percentage of rh Bri2 BRICHOS-AU1 that reached brain parenchyma using only MBs was higher (1% of the total protein administered) (**paper III**) than when it was administered without MBs (**paper I**) or with MBs and FUS (**paper II**). Altogether these results point to that rh Bri2 BRICHOS has a higher BBB permeability than reported for designed affibodies (343). Although the data obtained here regarding the internalization of BRICHOS domains in the brain and by neuronal cells in the brain hold unanswered questions, the findings open the possibility to explore the use of lipid MBs without ultrasound to potentially increase the uptake of therapeutic compounds into the CNS.

5.3 RH BRICHOS DOMAINS TREATMENT EFFECTS IN AD MOUSE MODELS (PAPER IV)

One of the most fundamental aspects of the studies on the BRICHOS domain is its ability to prevent the amyloid formation and neurotoxic effects of Aβ42 (17, 18, 233). Studies have shown that rh proSP-C BRICHOS domain is more potent than the monoclonal antibody Aducanumab *in vitro* as regards inhibition of Aβ42 oligomer formation (344). We aimed to uncover the therapeutic potential of a chronic treatment in AD mouse models using single point mutant monomer of rh Bri2 BRICHOS. This mutation is reported to shift the assembly equilibrium towards smaller species, making momomeric rh Bri2 BRICHOS R221E efficient in preventing Aβ42 neurotoxicity in mouse hippocampal slices (19). Before the treatment, we could confirm that rh Bri2 BRICHOS R221E permeates the BBB, as we observed for the wt version of rh Bri2 BRICHOS (**papers I-III**), and that its half-life in serum is longer than for any of the species of wt Bri2 BRICHOS.

We initially chose the "milder" mouse model App^{NL-F} aged 19 months and we expanded the study including a second mouse model with more aggressive Aβ pathology, App^{NL-G-F}, that was aged 3 months. During the treatment, the mice did not show any signs of general discomfort such as eg. weight loss, suggesting lack of immunological response, probably due to the high similarity between human and mouse BRICHOS. After rh Bri2 BRICHOS R221E administration, the study was divided in two experimental parts. First, we conducted a series of behavioral experiments to analyze if BRICHOS gave rise to improved cognitive function in these AD mouse models. There was an improvement in the physical activity of treated App^{NL-F} mice in open field compared to the non-treated group, approaching values obtained for aged-matched wt mice. Moreover, we estimated the ratio of rh Bri2 BRICHOS R221E/Aβ42 in App^{NL-F} mice and found that the behavioral effects obtained in these mice correlate well with the inhibition of γ oscillations in mouse hippocampal slices (19). Reduced hippocampal γ oscillations are associated with neurological disorders, and their stimulation attenuates AD-associated pathology in mouse models (345, 346). However, the performance of the treated mice in other cognitive tests did not show any significant difference compared to the control group. Given that fact, we performed behavioral analyses on App^{NL-G-F} mice, and we then observed that treated mice had significantly improved short-term memory and object recognition memory compared to controls.

In the second part of the study, we characterized the impact of the treatment on the Aβ associated effects using biochemical analyses. We determined that total levels of TRIS soluble and insoluble Aβ40 and Aβ42 measured by ELISA in hippocampus and cortex of the PBS and rh Bri2 BRICHOS R221E treated App^{NL-G-F} mice were similar, suggesting that rh Bri2 BRICHOS R221E treatment does not affect Aβ generation or its clearance. The main changes observed were for Aβ plaque load, astrogliosis and microgliosis, results that were qualitatively comparable with the ones obtained in APP/PS1 double transgenic mice that co-overexpress Bri2 or proSP-C BRICHOS (347). Despite biochemical analyses being performed four weeks after the last treatment, significant changes were observed. Mice treated with rh Bri2

BRICHOS R221E had significant lower levels of Aβ antibody and Thioflavin S positive plaques in the cortex.

Neuroinflammation is an important component of AD, and AD brains have shown notable activation of microglia and astrocytes in the vicinity of amyloid plaques that seems to increase the APP production and exacerbate Aβ burden (348, 349). Morever, App^{NL-F} mice have AD associated astrogliosis and microgliosis around the amyloid plaques. We therefore investigated possible changes in neuroinflammation affected by the treatment of rh Bri2 BRICHOS R221E. Reactive astrocytes have increased expression of glial fibrillary acidic protein (GFAP) and data shows that in early stages of AD, amyloid plaques are surrounded by an elevated number of GFAP positive astrocytes (350). Our analyses revealed a reduction of activated astrocytes and activated microglia surrounding Aβ plaques in App^{NL-F} treated mice. It is worth noting that most GFAP positive astrocytes appeared to be colocalized with plaques and that this colocalization was significantly reduced in the rh Bri2 BRICHOS R221E treatment group. The differences of activated microglia were only observed in the cortex region which could be explained with a milder microglial response in AD hippocampus (351).

Next, based on preclinical studies showing a negative correlation between activated microglia and cognition in AD patients (352), we addressed possible existing correlations between our data on Aβ deposition and microglia and astrocyte activation in the control and treatment groups. We observed a negative correlation between Thioflavin S positive plaques and Aβ burden in the control group that suggests the existence of different Aβ aggregate morphologies in different AD mouse models (353). In addition, the control mice showed positive correlation of Aβ plaque load with astrocytes and microglia markers as well as with Aβ and GFAP colocalization, while there is a trend of negative correlation of the latter with Thioflavin S positive plaques. This migth be explained by a decrease in the clearing ability of microglia due to an increment in number or size of plaques (354). Notably, the treatment of App^{NL-F} mice with rh Bri2 BRICHOS R221E eliminated these correlations, which suggest that the BRICHOS treatment exerted effects on both Aβ antibody and Thioflavin S positive plaques, and this led to a decrease in activated astrocytes and microglia.

Lastly, we believe that molecular chaperones able to cross the BBB, such as Bri2 BRICHOS, might be a potential strategy to find AD drug candidates. Up to date the antibodies that are being used in clinical trials have been focused on Aβ plaque reduction, and data is limited in terms of cognitive improvements in AD mouse models (Table 2) (200). Our data support that the inhibitory effects that rh Bri2 BRICHOS exert on the generation of neurotoxic species during the Aβ aggregation pathway *in vitro* are reproduced in the treated App^{NL-F} mice *in vivo*. In order to translate the treatment effects of rh Bri2 BRICHOS R221E observed in mouse models to an AD patient, we have extrapolated our *in vivo* and in *vitro* results to calculate the ratio between rh Bri2 BRICHOS and Aβ42 in the human brain, resulting in a reduction of 30 to 60% of the Aβ associated neurotoxicity, assuming BRICHOS BBB permeability is the same in humans as in mice.

To sum up, this work reports that repeated intravenous treatment with single mutant monomeric variant of rh Bri2 BRICHOS that can cross the BBB shows improvements in cognition, and decrease in inflammation and Aβ burden in App^{NL-F} mice.

Table 2. Selected preclinical trials of anti-amyloid-β monoclonal antibodies for AD and data from **paper IV**. Dashes indicate absence of information or unpublished data.

Drug	References	Aβ target	Animal model	Dose	Route of administration	Duration	Brain penetrance	Aβ plaques	Additional observation	Cognitive improvement
Bapineuzumab (3D6, murine precursor)	Bard F., et al., 2000 (330)	Fibrillar and soluble Aβ	PDAPP mice	-	Intraperitoneal injection	Weekly injections, for 24 weeks	-	86% reduction	Fc receptor-mediated microglial phagocytosis of Aβ	-
Solanezumab (m266, murine version)	DeMattos R.B., et al., 2001 (355)	Soluble and insoluble Aβ	PDAPP mice	500 µg	Intraperitoneal injection	Every 2 weeks for 20 weeks	-	Reduced levels of Aβ	-	A single injection reversed memory deficits
Gantenerumab	Bohrmann B., et al., 2012 (356)	Aβ fibrils	PS2APP mice	20 mg/kg	Intravenous injection via tail vein	Once a week, for 20 weeks	-	Reduced Aβ plaques	Microglial phagocytosis and prevention of plaque formation	No improvement in spatial memory test (MWM test)
Crenezumab (MABT5102A)	Adolfsson O., et al., 2012 (357)	Aβ monomers, oligomers and fibrils	hAPP$^{(V717I)}$/PS1 mice	10 mg/kg	Intraperitoneal administration	Once, twice or 14 times a week, for 9 weeks	-	Reduced by week 3 and in some animals, removed by week 9	Increased microglia uptake of Aβ oligomers	-
BAN2401 (mAb158, murine version)	Tucker S., et al., 2015 (358)	Soluble Aβ protofibrils	Tg-ArcSwe mice	10 mg/kg	Intraperitoneal injection	Once a week for 13 weeks	0.2%	Reduced protofibril levels by 42% in brain	-	-
Aducanumab	Sevigny J., et al., 2016 (359)	Aβ aggregates (soluble oligomers and insoluble fibrils)	Tg2576 mice	30 mg/kg	Intraperitoneal injection	Once a week for 24 weeks	C_{max} in brain was 1.062 ng/g tissue	Up to 70% reduction of all forms of Aβ plaques	-	-
Monomeric Bri2 BRICHOS R221E	Unpublished	Aβ fibrils	*App*$^{NL-F}$ and *App*$^{NL-G-F}$ mice	20, 10 mg/kg	Intravenous injection via tail vein	Two injections a week, for 10 weeks	0.4-0.5%	Reduced Aβ plaque deposition	Reduced activated microglia and astrocytes	Improved working and object recognition memory

6 CONCLUDING REMARKS

"No masterpiece was ever created by a lazy artist." -Salvador Dali

In summary, the work presented in this thesis aimed to elucidate, for the first time in mice, the therapeutic potential of rh Bri2 BRICHOS as a drug candidate for AD. Based on our main findings, we conclude that:

⇒ Rh Bri2 BRICHOS-AU1, but not rh proSP-C BRICHOS domain, can penetrate into the brain parenchyma after systemic injection. Moreover, after injection of different rh Bri2 BRICHOS assembly states, the mononers and dimers, but not oligomers, appeared to pass the BBB.

⇒ The BRICHOS domain from proSP-C and Bri2 have different pharmacokinetic properties and qualitatively different BBB permeabilities.

⇒ The delivery of rh proSP-C BRICHOS and rh Bri2 BRICHOS-AU1 is efficently enhanced by FUS in combination with MBs. Both rh BRICHOS domains reach the brain parenchyma and are internalized by a subset of neurons in the cortex and hippocampus, but only rh Bri2 BRICHOS-AU1 is found in both hemispheres after administraiton together with FUS in combination with MBs.

⇒ High amounts of rh Bri2 BRICHOS-AU1 are detected in the brain parenchyma after intravenous administration, representing about 0.4% of the total amount injected. Furthermore, even higher amounts of rh Bri2 BRICHOS protein are found in both hemispheres when only MBs and rh Bri2 BRICHOS-AU1 are used, and MBs promote also transfer of the fusion protein rh Bri2 BRICHOS-mCherry.

⇒ Intravenous treatment with monomeric rh Bri2 BRICHOS R221E in *App* knock-in mice improves working and recognition memory and reduces plaque deposition. In addition, the treatment reduces the presence of activated astrocytes and microglia as well as the activated astrocytes surrounding Aβ plaques.

⇒ Extrapolation of our *in vivo* data to potential effects of rh Bri2 BRICHOS R221E in AD patients indicates a significant decrease in Aβ42 associated toxicity.

7 POINTS OF PERSPECTIVE

"No limits but the sky."- Chapter XVI, Part I, Don Quixote. Miguel de Cervantes

The AD field is continuously evolving to understand the exact mechanisms underlying the disease and discover drugs that can delay or reverse the pathology. In this thesis we have added a piece to the large puzzle that constitutes this field with our evaluation of the molecular chaperone Bri2 BRICHOS as a potential treatment of AD based on its known anti-amyloid and anti Aβ toxicity properties.

The BBB is a rate-limiting factor and a great impediment for the success of pharmaceutical treatments. Therefore, there is a need to develop delivery systems or technologies that can facilitate potential drugs to reach their target in the brain. The results obtained in this thesis show that the brain uptake of rh Bri2 BRICHOS is higher than for antibodies used in clinical trials (272). Although we could for the first time find out that rh Bri2 BRICHOS cross to the brain parenchyma, there will be more studies needed to better clarify the underlying mechanisms by which Bri2 BRICHOS pass the BBB and is internalized by neurons.

The use of FUS and/or MBs represent a safe way to deliver drugs to the brain and, to enhance the uptake of rh Bri2 BRICHOS domains. The interesting discovery on the effects of MBs on rh Bri2 BRICHOS permeability enable us to explore if the same effects are present for other proteins. Considering the low number of mice analyzed, the effects need to be studied further.

There are some important research questions to answer in the near future. For instance, a more detailed investigation of the pharmacokinetics properties, clearance and uptake mechanisms of rh Bri2 BRICHOS or rh Bri2 BRICHOS R221E combined or not with MBs would be relevant for future treatment strategies using BRICHOS domains. It has been shown that plasma proteins have the ability to transfer from the periphery to the brain in healthy adult mice and that the transport mechanism varies with age (271). It would be useful to compare the brain permeability of rh Bri2 BRICHOS and plasma proteins in combination with MBs in animal models. A more detailed investigation on the brain distribution and quantification of rh Bri2 BRICHOS uptake and treatment by for instance PET imaging, could provide us useful *in vivo* information.

In this work we have demonstrated that we can use molecular chaperones in animal models of AD and reduce amyloid burden. However, there is research that needs to be done as regards parameters that could affect the outcome of preclinical studies, such as optimal dose and timepoints of the treatments, as well as means to increase the uptake and the retention of rh Bri2 BRICHOS in the brain.

We have observed clear effects of rh Bri2 BRICHOS R221E in neuroinflammatory markers in AD mouse models. Identifying the changes observed in microglia and astrocytes activation

after BRICHOS treatment by for instance looking at the gene expression levels of inflammation markers might provide us valuable answers about the AD related pathways affected by the treatment of Bri2 BRICHOS. Proteomic studies to characterize microglia in AD mouse models have identified a heterogeneity in the microglial Aβ response (360).

We also should consider the stage at which the animal should be treated in future studies. The treatment study presented in this work has been performed in mice with their brains loaded with amyloid plaques. It would be of interest to investigate rh Bri2 BRICHOS in mice with less advanced pathology as a prevention and to slow down or stop the amyloid cascade.

Our ultimate goal would be to translate these results into humans, therefore, a next step to study non-human primates should be considered. Rh Bri2 BRICHOS purification and expression at large scale is still a technical limitation that needs to be improved. However, there is ongoing research addressing this matter. An alternative approach to rh Bri2 BRICHOS administration could be to increase the expression of endogenous Bri2 BRICHOS in the brain, initially in mouse models.

We believe that our results provide the bases for further exploration of Bri2 BRICHOS as a new therapeutic strategy particularly for AD. Hopefully, we will move forward to the treatment of AD and other neurodegenerative diseases.

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